Aspects of Mole-Rat Neurobiology (Rodentia: Bathyergidae) with Particular Reference to Reproductive Suppression, Sociality and Neurogenesis in Eusocial Species

Zhou, Shuzhi

Awarding institution:
King's College London

Aspects of mole-rat neurobiology (Rodentia: Bathyergidae); with particular reference to reproductive suppression, sociality and neurogenesis in eusocial species

by SHUZHI ZHOU

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Reproduction and Endocrinology, School of Biomedical and Health Sciences

King's College London, University of London
London, UK

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Supervisors: Professor C.W. Coen, King's College London, ULU
Dr C.G. Faulkes, Queen Mary, ULU
Dr P.D. Taylor, King's College London, ULU

ABSTRACT

Mole-rats (of the Bathyergidae family) provide a unique taxonomic group for studying many aspects of neurobiology. In this family, ecological constraints have led to diverse social and reproductive strategies. Eusociality occurs in two species of Bathyergidae rodents; naked mole-rats (*Heterocephalus glaber)* and Damaraland mole-rats (*Fukomys damarensis)*. The females of these two eusocial species are at the extreme end of the socially-induced infertility continuum whereby ovulation is physiologically blocked. This family provides an ideal model to glean insight into the neurobiological mechanisms of reproductive suppression, eusocial behaviour and neuroplasticity. In this thesis, I report that: (1) naked mole-rats display substantial numbers of kisspeptin-immunoreactive cell bodies in the rostral periventricular region of the third ventricle, paraventricular hypothalamic nucleus, arcuate nucleus and dorsomedial nucleus, irrespective of reproductive state, sex or presence of gonads, (2) reproductive naked mole-rats display significantly higher number of kisspeptin-immunoreactive cell bodies in the rostral periventricular region of the third ventricle and paraventricular hypothalamic nucleus than subordinates, (3) subordinate Damaraland mole-rats have an absence of a RFamide neuronal population that is found in abundance in reproductive Damaraland mole-rats, with no effect of gonadectomy or sex differences, (4) the presence of a sexually monomorphic population of hypothalamic tyrosine hydroxylase neurones indicates the lack of sexual differentiation in the brains of naked mole-rats, (5) the shared occurrence of oxytocin and its receptor in the nucleus accumbens of both eusocial naked mole-rats and eusocial Damaraland mole-rats indicates the possibility of convergent evolution in this family, (6) two novel locations for vasopressin receptor binding in the brains of Damaraland mole-rats have evolved independently during the divergence of this species from a common ancestor, and (7) naked mole-rats have a remarkably low rate of adult hippocampal neurogenesis, a low rate that is shared with other long-lived, group-living animals.

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This project was cleared by the Ethics Committee of King's College London, University of London.

GLOSSARY AND DEFINITIONS

3V	third ventricle
AC	anterior commissure
AHN	anterior hypothalamic nucleus
AR	androgen receptor
Arc	arcuate nucleus
AVP	arginine vasopressin
AVPV	anteroventral periventricular nucleus
AVT	arginine vasotocin
BNST	bed nucleus of the stria terminalis
cArc	caudal arcuate nucleus
BrdU	5-bromo-2'-deoxyuridine
CNS	central nervous system
DCX	doublecortin
DMH	dorsomedial hypothalamic nucleus
DG	dentate gyrus
ERα	oestrogen receptor α
ERβ	oestrogen receptor β
FSH	follicle stimulating hormone
GCL	granule cell layer
GDX	gonadectomised
GnIH	gonadotrophin-inhibitory hormone
GnRH-1	gonadotrophin-releasing hormone-1
GPR54	G protein-coupled receptor 54
HDB	horizontal limb of the diagonal band
HPG	hypothalamo-pituitary-gonadal axis
ic	internal capsule
ICj	islands of Calleja
ir	immunoreactive
KO	knockout
LH	luteinising hormone
LS	lateral septum
LV	lateral ventricle
MBH	medial basal hypothalamus
ME	median eminence
MEex	external zone of the median eminence
MEin	internal zone of the median eminence

MeA	medial amygdala
ML	molecular layer
MM	medial mammillary nucleus
mPFC	medial prefrontal cortex
MPO	median preoptic nucleus
MPOA	medial preoptic area
MS	medial septum
mt	mammillothalamic tract
NAcc	nucleus accumbens
OB	olfactory bulb
opt	optic tract
OT	oxytocin
OTR	oxytocin receptor
OV	olfactory ventricle
OVLT	organum vasculosum of the lamina terminalis
PBS	phosphate buffered saline
PC	piriform cortex
PeN	periventricular nucleus
PFA	paraformaldehyde
POA	preoptic area
PS	pituitary stalk
PT	pars tuberalis
PVH	paraventricular hypothalamic nucleus
PVT	paraventricular thalamic nucleus
rArc	rostral arcuate nucleus
rAVPV	rostral anteroventral periventricular nucleus
RCh	retrochiasmatic nucleus
Re	reuniens thalamic nucleus
RFRP-1	RFamide-related peptide-1
RFRP-3	RFamide-related peptide-3
RMS	rostral migratory stream
RP3V	rostral periventricular region of the third ventricle
SCN	suprachiasmatic nucleus
SDN-POA	sexually dimorphic nucleus of the preoptic area
SEM	standard error of the mean
SGZ	subgranular zone
SON	supraoptic nucleus
SNB	spinal nucleus of the bulbocavernosus

SNpc	substantia nigra pars compacta
SVZ	subventricular zone
TH	tyrosine hydroxylase
V1aR	vasopressin V1a receptor
VDB	vertical limb of the diagonal band
VLS	ventrolateral septum
VMH	ventromedial hypothalamic nucleus
VP	ventral pallidum
VTA	ventral tegmental area
ZI	zona incerta

Definitions

The terms ‘social status’, ‘reproductive status’ and ‘breeding status’ are used interchangeably and all have the same meaning. The terms ‘non-reproductive’ and ‘subordinate’ are used interchangeably when describing mole-rats and refer to the same status. Reproductively-activated mole-rats are subordinates that have been paired together with an opposite sex mate and show physical and behavioural signs of fertility-onset, but are non-breeders. Breeding mole-rats are reproductively-activated mole-rats who have produced at least one litter.

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General introduction

- **Cooperative breeding and eusociality**

Cooperative breeding occurs in a number of mammals (e.g. marmosets, meerkats, wolves, mongooses, prairie voles and mole-rats) (Faulkes and Bennett, 2001). In cooperatively breeding mammals, individuals other than the parents help care for offspring. Also, reproduction is partitioned unequally among members of the social group (termed 'reproductive skew'), with socially dominant individuals monopolising the breeding opportunities (Clutton-Brock, 2002). The extent of cooperation varies from social groups where several males and females regularly breed, to social groups where reproduction is restricted to a single breeding female and one or two dominant males. This reproductive control ranges from infanticide of the offspring of other subordinates, to interference by dominants with subordinate mating attempts, and to actual suppression of subordinate reproductive physiology (Faulkes and Abbott, 1997).

Eusociality is a social system typically associated with ants, termites, bees and wasps where only a few individuals within a large colony engage in direct reproduction, while other members are sterile and act to support the reproductive efforts of the colony as a whole. An eusocial breeding strategy is considered to be an evolutionarily advanced level of colonial living where groups of cooperatively breeding conspecifics have a reproductive division of labour, cooperative care of young and more than two overlapping adult generations (Wilson and Holldobler, 2005). On the eusociality continuum, social systems are rated on a scale from zero to one, such that in societies with a low skew (close to zero), all individuals have almost equal opportunities for breeding and those with a high skew (close to one) have unequal opportunities for breeding (Sherman *et al.*, 1995).

This eusocial system initially posed a threat to Charles Darwin's theory of evolution through natural selection, which emphasised the relative fitness of individuals in determining traits that would be transmitted across generations (Darwin, 2008). It was difficult to understand how the trait of sterility (a negative trait) could be transmitted to future generations through natural selection. This dilemma has been resolved largely through the insights of Hamilton who analysed the factors that contribute to inclusive fitness; genes that lead to sterility in some individuals can enhance fitness if those individuals support the reproductive efforts of their close genetically related relatives who are likely to carry copies of the same genes (Hamilton, 1964a,b). By helping closely related individuals rear their offspring, helpers gain indirect fitness benefits and increase their own chances of becoming breeders by gaining experience (Emlen, 1997). Thus, non-reproductive animals may delay their dispersal until opportunities arise, when environmental conditions are favourable or unrelated animals are present, or they have gained sufficient skills to ensure successful independent reproduction.

- **African mole-rats**

Eusociality occurs in two species of Bathyergidae rodents; naked mole-rats, *Heterocephalus glaber*, and Damaraland mole-rats, *Fukomys damarensis* (previously known as *Cryptomys damarensis*, some research papers still use the old nomenclature). African mole-rats are subterranean hystricomorph rodents endemic to sub-Saharan Africa. The family Bathyergidae is composed of 18 or more species and six genera (Faulkes and Bennett, 2001; Ingram *et al.*, 2004). Of the six genera, three genera (*Georychus*, *Heliophobius* and *Bathyergus*) are solitary and three genera (*Fukomys*, *Cryptomys* and *Heterocephalus*) are social, displaying varying degrees of sociality and cooperative breeding (Figure 1.1). Amongst solitary mole-rats, interactions with conspecifics are restricted to transient mating and short-lived contacts between mother and pups or between littermates (Bennett and Jarvis, 1988). In contrast, social mole-rat species exhibit a reproductive division of labour; where reproduction in colonies is highly skewed and subordinate animals have little chance of ever reproducing (Faulkes and Bennett, 2001).

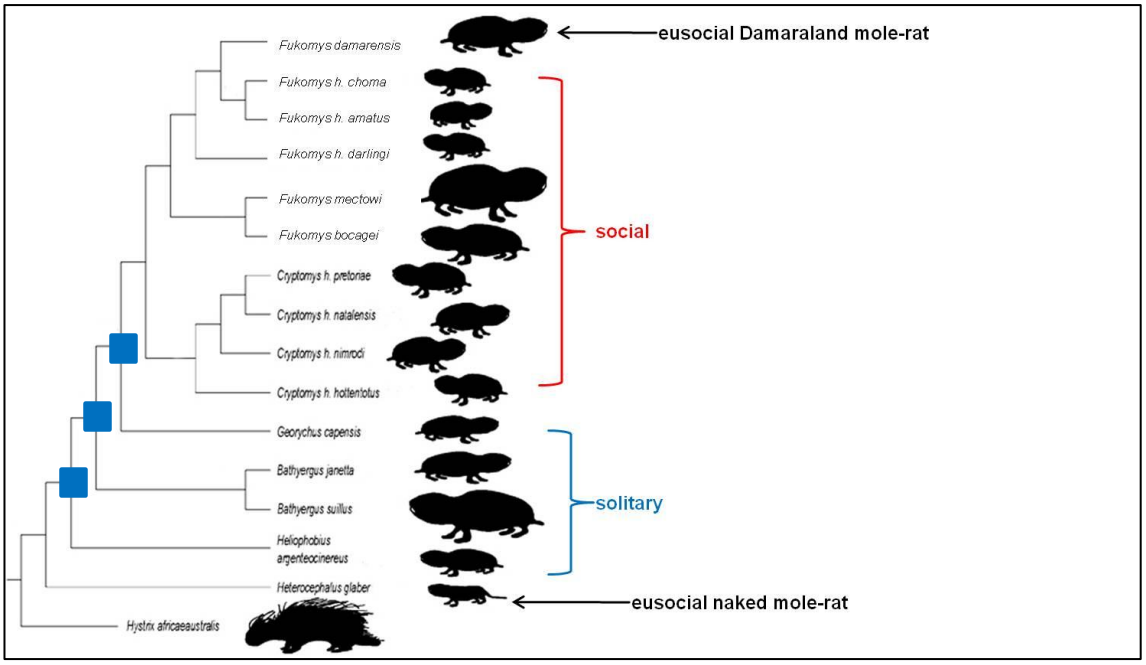


Figure 1.1: A schematic diagram showing the evolutionary tree of sociality in the Bathyergidae family from a common ancestor. Blue nodes lead to solitary species. The blue parenthesis indicates solitary species and the red parenthesis indicates social species. Note that the eusocial naked mole-rat at the base of the tree and the eusocial Damaraland mole-rat at the top of the tree are separated by a number of common ancestors that vary in degrees of sociality. From the eusocial naked mole-rat at the base of the tree, sociality was lost, and then evolved again. Eusociality has evolved independently twice within the same family. Modified from Faulkes *et al.*, 2004.

Two species, the naked mole-rat (*Heterocephalus glaber*) and the Damaraland mole-rat (*Fukomys damarensis*) are considered to be eusocial (Jarvis and Bennett, 1993). Previous molecular phylogenetic studies based on mitochondrial DNA analysis have indicated that the two eusocial mole-rat species (naked and Damaraland mole-rats) are evolutionarily very divergent (Figure 1.1) (Jarvis and Bennett, 1993; Faulkes *et al.*, 1997). Comparisons of the phylogeographical patterns of genetic divergence in African mole-rats have shown that: (a) divergence of the *Heterocephalus* lineage from the common ancestor in East Africa occurred 40–48 million years, (b) radiation of the *Heliophobius* lineage from East Africa into South Africa occurred 34–40 million years ago, with some populations crossing the Rift Valley (a physical barrier), (c) *Georychus* and *Bathyergus* lineages diverge in South Africa 22–26 million years ago, and finally (d) *Cryptomys/Fukomys* diverges spreading north into Central Africa 14–17 million years ago, although it is not known when the *Fukomys* genus diverged from the *Cryptomys* genus (Figure 1.2) (Faulkes *et al.*, 2004; Kock *et al.*, 2006). Mole-rats of the genus *Heterocephalus* (including the eusocial naked mole-rat) are eusocial; *Heliophobius, Bathyergus* and *Georychus* mole-rats are solitary; mole-rats of the genus *Cryptomys* are social and mole-rats of the genus *Fukomys* (including the eusocial Damaraland mole-rat) are also social. These studies have indicated that the eusocial naked mole-rat, *Heterocephalus glaber* is basal and divergent in the family, separated from the social *Fukomys* genus by the three solitary genera, leading to the conclusion that either sociality or solitarity has been gained or lost more than once during their adaptive radiation though sub-Saharan Africa (Faulkes *et al.*, 1997; Faulkes *et al.*, 2004).

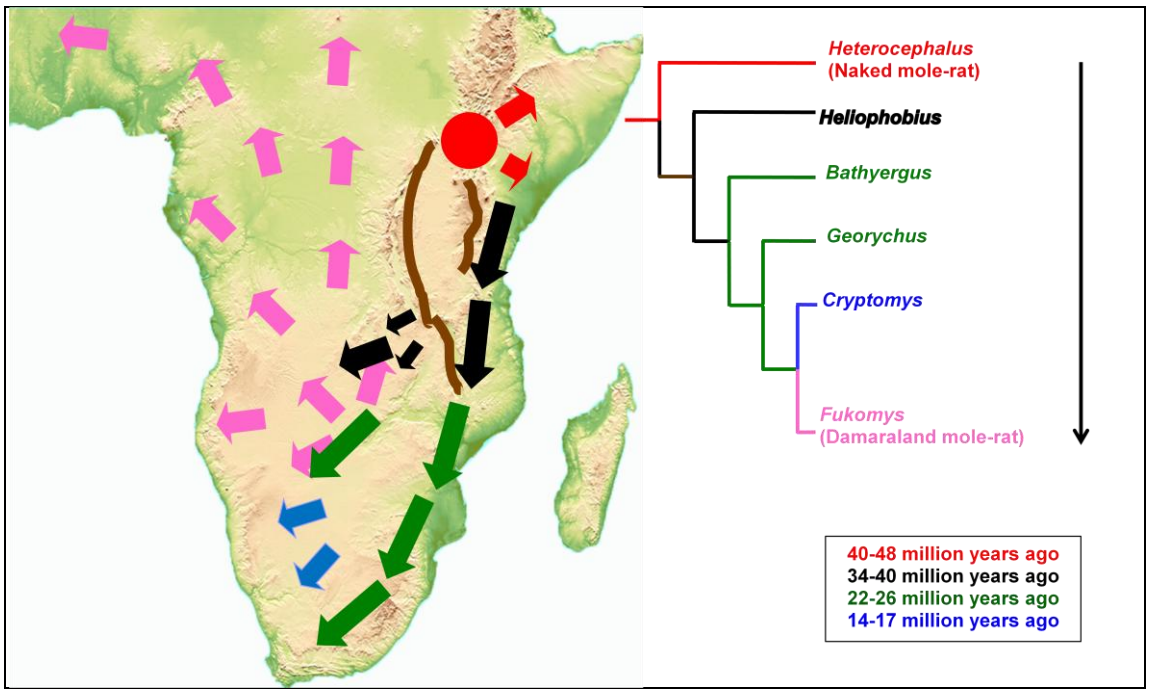


Figure 1.2: A schematic diagram showing the phylogeographical patterns of genetic divergence and speciation in African mole-rats (Family: Bathyergidae) in the context of rift valley formation in Africa (indicated by brown lines). African mole-rats are distributed in a range of habitats across sub-Saharan Africa, and display a range of social and reproductive strategies. Both molecular systematic studies of Rodentia and the fossil record of bathyergids indicate an ancient origin for the family. Mole-rats of the genus *Heterocephalus* are indicated by red arrows and diverged 40-48 million years ago; mole-rats of the genus *Heliophobius* are indicated by black arrows and diverged 34-40 million years ago; mole-rats of the genera *Georychus* and *Bathyergus* are indicated by green arrows and diverged 22-26 million years ago; mole-rats of the genus *Cryptomys* are indicated by blue arrows and diverged 14-17; mole-rats of the genus *Fukomys* are indicated by pink arrow and it is not known when this genus diverged. While early divergences may have been independent of rifting (indicated by the central black arrows), patterns of distribution of later lineages may have been influenced directly by physical barriers imposed by the formation of the rift valley, and indirectly by accompanying climatic and vegetative changes. The genus *Fukomys* appears to have undergone an extensive radiation and shows the widest geographical distribution. Modified from Faulkes *et al.*, 2004.

Comparisons between group size and the environment where different species exist has led to the idea that the degree of sociality observed across the family Bathyergidae is correlated with the aridity of the habitat and subsequent food availability (Figure 1.3) (Jarvis *et al.*, 1994; Faulkes *et al.*, 1997). The aridity food distribution hypothesis has been proposed to explain the subsequent cost and risks associated with foraging and dispersal in arid areas (Jarvis *et al.*, 1994). A major cost associated with fossorial life is the extraordinary amount of energy required to move to new territory during foraging. This is particularly significant for fossorial species that inhabit environments where food is patchily distributed, as is the case for both the eusocial naked and Damaraland mole-rats (Bennett and Faulkes, 2000a). In addition, both species consume geophytes, underground roots and tubers that are only exposed by burrowing, and burrowing is difficult during the dry season when the soil is hard and packed. A large number of animals working together may be required for success in this niche since this is will increase the probability of encountering a localised food patch and reduce energetic costs. By contrast, solitary species are found in mesic habitats where food resources are evenly distributed and the soil is readily workable, where a single animal can easily find sufficient food to sustain itself (Jarvis and Bennett, 1993; Jarvis *et al.*, 1994).

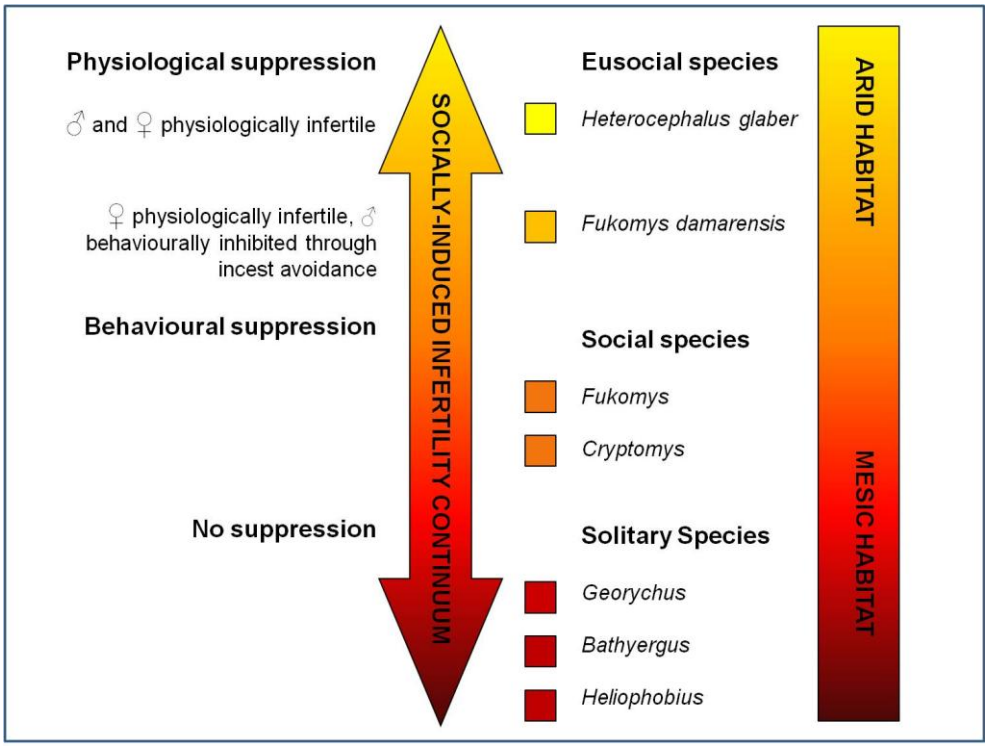


Figure 1.3: Schematic diagram of species of the family Bathyergidae assembled according to the degree of sociality displayed and type of habitat in which they occur. ♀= female, ♂= male. Modified from Van de Walt, 2003.

- **Reproductive regulation in mammals**

In mammals, the hypothalamo-pituitary-gonadal (HPG) axis regulates reproduction and this axis is controlled by the central nervous system (CNS) which receives both internal and external stimuli to drive puberty-onset. Gonadotrophin-releasing hormone-1 (GnRH-1) neurones are central to gonadal and behavioural maturation. GnRH-1 regulates the synthesis and secretion of pituitary gonadotrophins, thereby providing a link between the neural and endocrine systems (Sisk and Foster, 2004).

GnRH-1 is a decapeptide produced in and secreted by specialised GnRH-1 neurones in the forebrain (Clarke, 1987). The afferents of these neurones project into the median eminence (ME) of the hypothalamus where they intermittently secrete pulses of GnRH-1. GnRH-1 is released into the pituitary portal system to the anterior pituitary where it stimulates the synthesis and release of the pituitary gonadotrophins: luteinising hormone (LH) and follicle stimulating hormone (FSH). Blood-borne LH and FSH then act on target cells in the gonads (testes and ovaries) to drive the production of egg and sperm cells, as well as the secretion of steroid hormones (Sisk and Foster, 2004). In spontaneously ovulating species, GnRH-1 is released from the ME in a pulsatile manner that leads to the cyclical synthesis and release of gonadal hormones, generating a continuous reproductive cycle. In contrast, in animals exhibiting induced ovulation, external stimulation is required to trigger GnRH-1 secretion and the preovulatory LH surge. Such stimulation is usually received from coitus (Bakker and Baum, 2000). Within the brain, steroids influence GnRH-1 secretion via neuroendocrine feedback loops. Modulation of the GnRH-1 pulse frequency is the primary mechanism by which the body alters its reproductive status during development.

In female animals, FSH released from the pituitary is responsible for follicular development in the ovaries. The maturing follicle secretes oestrogen and a small amount of progesterone. During development, the maturing follicle secretes higher levels of oestrogen and an absence of progesterone. This causes the GnRH-1 pulse frequency to increase, the rise in GnRH-1 release triggers a surge in LH that induces ovulation (Clarke and Pompolo, 2005). The follicle ruptures and the oocyte is released into the fallopian tube. The remnants of the follicle develop into a corpus luteum that secretes progesterone and oestrogen. These hormones are required to develop and maintain the endometrium for implantation and growth of the embryo. The elevated levels of progesterone and oestrogen cause the GnRH-1 pulse frequency to decrease, and inhibit LH and FSH release. In response to the diminishing level of plasma LH, the oestrogen and progesterone decline and the negative feedback to the pituitary terminates (Clarke, 1995) (Figure 1.4A).

In males, LH and FSH regulate spermatogenesis. FSH initiates sperm production while LH stimulates the production of testosterone in the interstitial cells.

Under the influence of testosterone, immature sperm cells develop into mature spermatozoa and are stored in the epididymus. Males do not produce a LH surge due to insufficient levels of oestrogen (Parvizi, 2000) (Figure 1.4B).

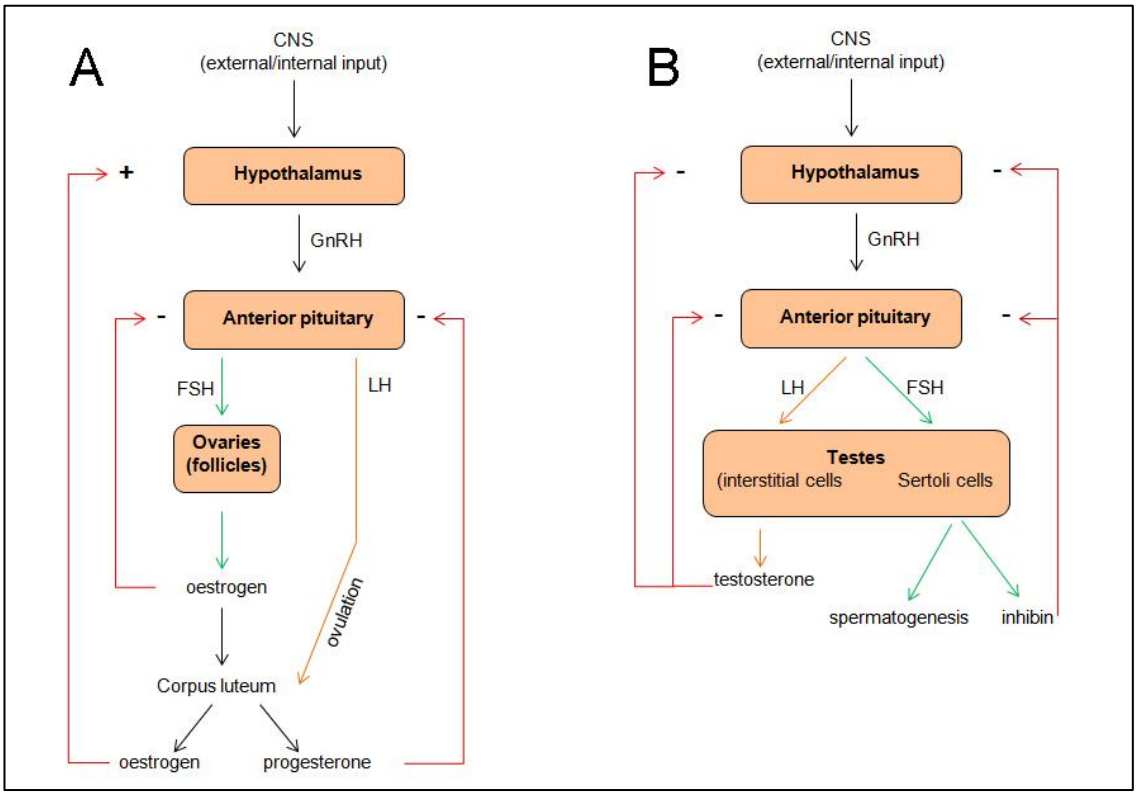


Figure 1.4: Schematic representation of the regulation of the hypothalamo-pituitary-gonadal axis in (a) females and (b) males. Modified from Oosthuizen, 2007.

On a parallel level, puberty onset also results in the maturation of sexual behaviour. In females, oestradiol and progesterone facilitate proceptive and receptive behaviours that signal readiness to mate (Blaustein and Erskine, 2002). In males, testosterone and its metabolites facilitate sexual motivation and copulatory behaviour (Hull *et al.*, 2002). During the critical period spanning late embryonic to early neonatal development, sexually differentiated neural circuits are programmed to mediate adult female or male reproductive behaviour. In adulthood, steroid hormones facilitate reproductive behaviour by eliciting cellular responses within the previously sexually differentiated neural circuits. The irreversible changes in CNS structure and the programming of adult behavioural responses to hormones caused by exposure to steroids during early neural development are called the 'organisational effects'; the facilitation of reversible reproductive behaviour by steroids in the adult is called an 'activational effect' (Morris *et al.*, 2004).

In most mammals, transient activation of the HPG axis during late prenatal or early postnatal life results in an increase in circulating gonadal steroids. These steroids

then participate in sexual differentiation and other programming of the CNS. GnRH-1 secretion soon declines throughout the prepubertal period, which may last for several years. After this period of quiescence, puberty begins when GnRH-1 secretion once again increases in frequency and remains high to stimulate gonadotrophin and steroid hormone secretion, this time, resulting in complete gonadal and sexual maturation (Sisk and Foster, 2004). However, what is not known is what triggers the timing of frequent GnRH-1 secretion, ultimately driving the pubertal transition.

Determining the neural mechanisms underlying the episodic secretion of GnRH-1 has proved to be a major technical challenge. Firstly, GnRH-1 neurones are very few in number, with only 1000-3000 cells found in various mammalian species. Secondly, GnRH-1 cell bodies are widespread throughout the brain. GnRH-1 neurones arise from the nasal placode region during early embryonic development where they migrate into the brain and spread diffusely throughout the vertical diagonal bands of Broca (VDB) and horizontal diagonal bands of Broca (HDB), medial septum (MS), organum vasculosum of the lamina terminalis (OVLT), preoptic area (POA) and mediobasal hypothalamus (MBH). Finally, these diffusely spread GnRH-1 neurones are able to fire synchronously even though these neurones have few innervations (Parvizi, 2000).

Various candidates (including melatonin, body fat and leptin) have been implicated in permitting puberty onset (Ebling and Cronin, 2000). The consequences of puberty, such as the defence of territory or mate, pregnancy and care of young are energetically expensive. Hence, when an individual goes through puberty, it must determine whether its body has grown sufficiently, whether the conditions are optimal to raise offspring, and what its relationship to other individuals are. For example, hormones such as leptin and insulin serve as important signals for metabolic fuel availability. Leptin receptors in the hypothalamus relay metabolic information to the HPG axis and permit high-frequency GnRH-1 release when these signals reach appropriate levels (Foster and Nagatani, 1999; Cheung *et al.*, 2001; Schneider, 2004). For seasonal breeders, the photoperiod signals the optimal time for puberty onset. The circadian clock in the suprachiasmatic nucleus (SCN) measures day length by controlling melatonin production in the pineal gland. Melatonin receptors in the hypothalamus relay this signal to the HPG axis permitting GnRH-1 release (Foster *et al.*, 1988; Ebling and Foster, 1989). In other species, sensory cues from conspecifics, such as mating or contact with an opposite sex, permit the onset of GnRH-1 at puberty (Bronson and Maruniak, 1975; Rissman, 1992).

So rather than a single factor triggering puberty, multiple permissive signals determine the precise timing of puberty onset. These signals are integrated in a species-specific manner to allow (or not allow) the pubertal increase in GnRH-1 pulse frequency. What remains largely unknown is what this integration mechanism is and

whether a master integrator even exists. Nevertheless, the correct combinations of permissive signals cannot explain the timing of puberty since these signals are not unique to this period of life. Thus, it is more probable that a developmental clock times puberty onset.

- **Kisspeptin and fertility**

Until recently, studies on the neuroendocrine control of reproduction in mammals have been limited to the hypothalamic GnRH-1 system. Kisspeptin and its receptor have emerged as key players in the regulation of reproduction. In 2001, four independent groups identified kisspeptin (product of *Kiss1* gene) as a high-affinity RFamide (Arg-Phe-NH_2) peptide ligand for the then orphan G protein coupled receptor 54 (GPR54), the kisspeptin receptor (Clements *et al.*, 2001; Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). Kisspeptins comprise a family of neuropeptides that are derived from the *Kiss1* gene. The initial product of the *Kiss1* gene (*KISS-1* in humans) is a 145-amino-acid peptide which is cleaved into a 54-amino-acid peptide (kisspeptin-54). There are also shorter peptides (kisspeptin-10, -13 and -14) that share a common RF-amidated motif with kisspeptin-54; collectively, they are termed kisspeptins (West *et al.*, 1998) (Figure 1.5). All four peptides (kisspeptin-10, -13, -14 and -54) exhibit the same affinity and efficacy for the kisspeptin receptor GPR54, indicating that the C-terminal end of the peptide is responsible for the binding and activation of GPR54 (Kotani *et al.*, 2001).

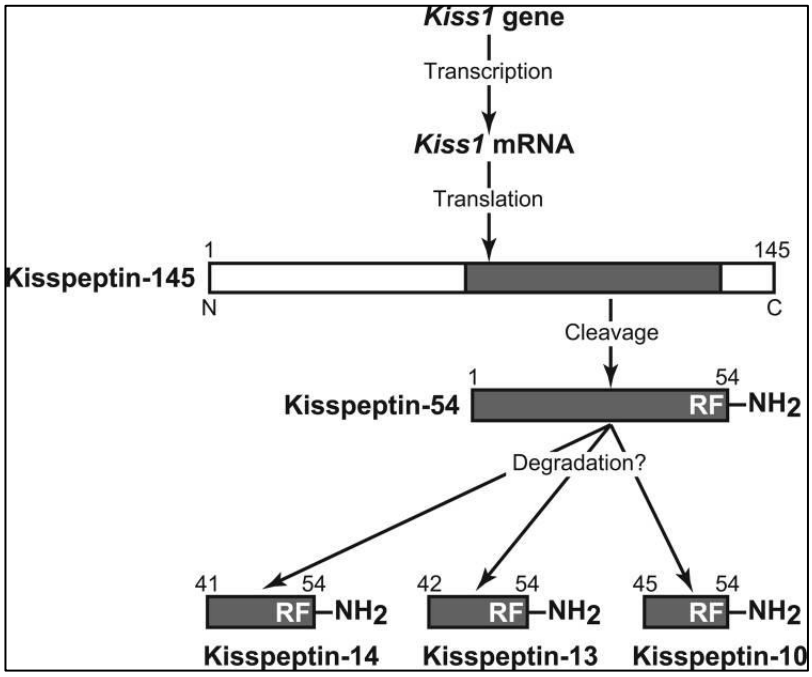


Figure 1.5: Products of the *Kiss1* gene. *Kiss1* mRNA is transcribed from the *Kiss1* gene and translated to form a 145-amino-acid propeptide called kisspeptin-145. Shown are the cleavage sites on the propeptide kisspeptin-145 that lead to the production of the RF-amidated

kisspeptin-54. Shorter peptides (kisspeptin-10, -13 and -14) share a common C terminus and RF-amidated motif with kisspeptin-54. No cleavage sites have been identified on the propeptide, it has been suggested that the shorter peptides may be degraded products of kisspeptin-54. Modified from Popa *et al.*, 2008.

Human kisspeptin-54 was originally known as 'metastin' and was found to inhibit cancer metastasis (Harms *et al.*, 2003). In 2003, kisspeptin-GPR54 signalling piqued the interest of reproductive endocrinologists when two independent research groups reported that mutations in the *GPR54* gene were associated with the idiopathic hypogonadotrophic hypogonadism and impaired pubertal maturation in human patients (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Unlike patients with Kallmann's syndrome, patients with homozygous *GPR54* mutations are normosmic, indicating that there are no major deficits in the embryonic migration of either olfactory neurones or GnRH-1 neurones (Schwanzel-Fukuda, 1999). Moreover, mice of both sexes bearing targeted deletions of *Gpr54* fail to undergo puberty and exhibit extreme deficiencies in reproductive function (Funes *et al.*, 2003; Seminara *et al.*, 2003). *Gpr54* knockout (KO) males have small gonads, low levels of testosterone, have disrupted spermatogenesis and do not exhibit mounting behaviour. Like humans with similar mutations, *Gpr54* KO mice have lower plasma levels of LH and FSH than do their wild-type counterparts (Seminara *et al.*, 2003). Nevertheless, some gonadotrophin secretion remains, indicating that pituitary gonadotrophins are still functional in *Gpr54* KO mice. Furthermore, the number of hypothalamic GnRH-1 neurones is normal in *Gpr54* KO mice in comparison to normal mice, which suggests normal GnRH-1 neuronal migration and synthesis (Messager *et al.*, 2005). Also, female *Gpr54* KO mice show an increase in LH and FSH in response to a series of GnRH-1 injections, as do humans (Seminara *et al.*, 2003; Messager *et al.*, 2005). Finally, mice bearing targeted deletions of the *Kiss1* gene have a similar phenotype to *Gpr54* KO mice (d'Anglemont de Tassigny *et al.*, 2007).

Several lines of evidence suggest that kisspeptin stimulates GnRH-1 secretion by acting through GPR54:

1. Central or peripheral administration of kisspeptin stimulates GnRH-1 and gonadotrophin secretion in rodents, sheep, monkeys and humans (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Matsui *et al.*, 2004; Thompson *et al.*, 2004; Dhillo *et al.*, 2005; Messager *et al.*, 2005; Shahab *et al.*, 2005; Dhillo *et al.*, 2007).
2. Central administration of kisspeptin increases GnRH-1 secretion in wild-type mice, but not *Gpr54* KO mice (Messager *et al.*, 2005).

3. Pre-treatment with GnRH-1 antagonists prevents kisspeptin-induced gonadotrophin secretion (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Matsui *et al.*, 2004; Shahab *et al.*, 2005).
4. The majority of GnRH-1 neurones express *Gpr54* (Irwig *et al.*, 2004; Han *et al.*, 2005; Messager *et al.*, 2005).
5. Kisspeptin-immunoreactive (-ir) fibres are found in close association with GnRH-1 neurones (Kinoshita *et al.*, 2005; Clarkson and Herbison, 2006; Decourt *et al.*, 2008; Smith *et al.*, 2008).
6. Expression of the immediate-early gene product Fos occurs in GnRH-1 neurones after central administration of kisspeptin in rodents (Irwig *et al.*, 2004; Matsui *et al.*, 2004).
7. Kisspeptin-10 depolarises GnRH-1 neurones in current-clamp recordings and generates inward current in voltage-clamp recordings (Han *et al.*, 2005; Pielecka-Fortuna *et al.*, 2008).

Animals employ various strategies to optimise reproductive success, including timing reproduction to an ideal birth season and timing ovulation to occur within a narrow window to maximise mating success. These different mating strategies are reflected in the unique and diverse organisation of the *Kiss1* neural circuitry. Some aspects of kisspeptin anatomy and physiology are unique to a particular species:

1. **Mouse (*Mus musculus*)**. In mice, *Kiss1* mRNA and kisspeptin-ir cell bodies are located in areas of the hypothalamus implicated in the neuroendocrine regulation of gonadotrophin secretion; including the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN) and the arcuate nucleus (Arc). Together, the AVPV and PeN are known as the rostral periventricular region of the third ventricle (RP3V). In addition, some cells expressing *Kiss1* mRNA are located in the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) (Gottsch *et al.*, 2004; Clarkson and Herbison, 2006). A population of kisspeptin-cell bodies were identified in the dorsomedial hypothalamic nucleus (DMH). There is a remarkable sex difference in the number of cell bodies in the RP3V region (consisting of the AVPV and PeN), with adult females exhibiting a 10-fold greater number of kisspeptin-ir cell bodies than males (Clarkson and Herbison, 2006).
2. **Hamster (Syrian, *Mesocricetus auratus*; and Siberian, *Phodopus sungorus*).** *Kiss1* mRNA (and its peptide product) is expressed in the Arc of Syrian hamsters raised in long-day photoperiod (Revel *et al.*, 2006). In the Siberian hamster, kisspeptin-ir cell bodies have been observed in both the AVPV and the Arc (Greives *et al.*, 2007; Mason *et al.*, 2007).

3. **Rat (*Rattus norvegicus*).** Kisspeptin-ir cell bodies were observed in the AVPV and Arc after colchicine treatment (Adachi *et al.*, 2007). *Kiss1* is expressed in the AVPV, PeN and Arc of rats (Irwig *et al.*, 2004; Smith *et al.*, 2006; Adachi *et al.*, 2007; Kauffman *et al.*, 2007). There is a remarkable sex difference in the number of *Kiss1* expressing cells in the RP3V (AVPV and PeN) region, where there are about 25 times more *Kiss1* cells in adult females compared to males (Kauffman *et al.*, 2007).
4. **Sheep (*Ovis aries*).** Kisspeptin-ir cell bodies are observed in the medial preoptic area (MPOA), DMH and Arc (Franceschini *et al.*, 2006; Pompolo *et al.*, 2006). The functional significance of kisspeptin-ir cells in the MPOA is unknown since GnRH-1 neurones in the MPOA do not express *Kiss1* (Smith *et al.*, 2008).
5. **Rhesus macaque (*Macaca mulatta*)**. Kisspeptin-ir cell bodies have been identified in the Arc/ME but not in the POA or AVPV. In the ME, kisspeptin and GnRH-1 axons are extensively associated, indicating that kisspeptin may regulate GnRH-1 secretion nonsynaptically at the level of the ME (Ramaswamy *et al.*, 2008).

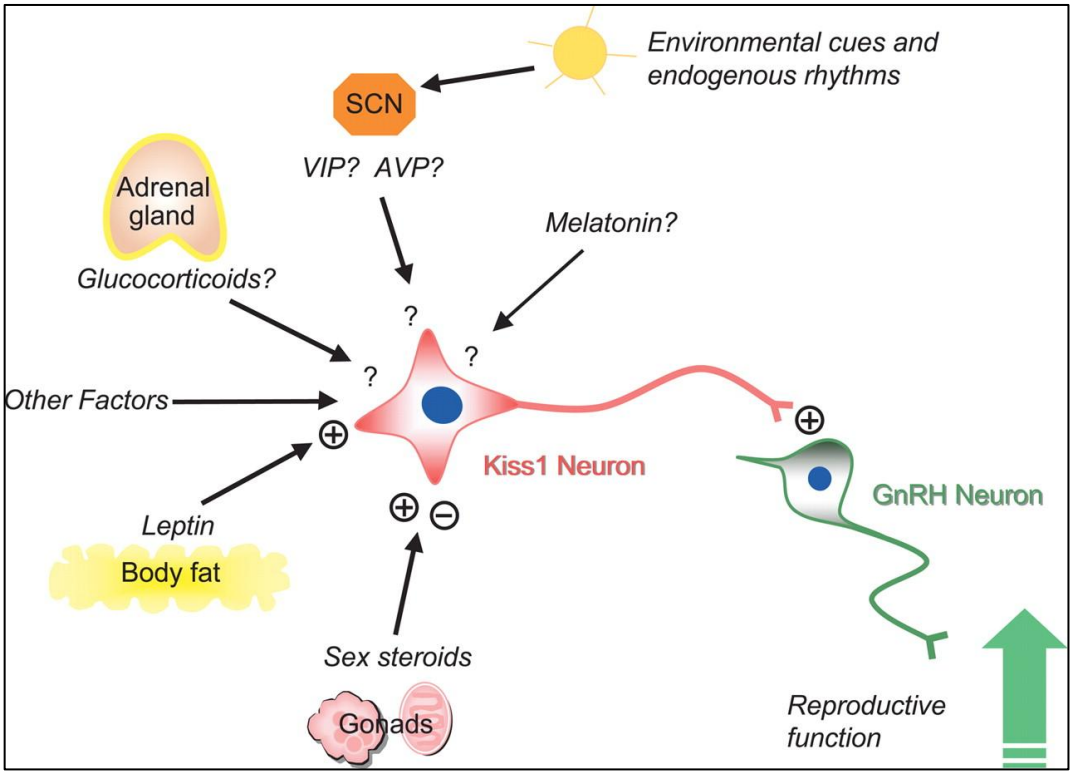


Figure 1.6: Kisspeptin neurones may act as central processors for relaying metabolic and environmental signals from the periphery to GnRH neurones. Kisspeptin stimulates GnRH secretion, and kisspeptin is both negatively and positively regulated by sex steroids. Modified from Dungan *et al.*, 2006.

The control of kisspeptin neurones emanates from a variety of sources, including steroid hormone feedback, metabolic signals and photoperiodic cues (Figure 1.6). The majority of *Kiss1* neurones express oestrogen receptor-α (ERα) and –β (ERβ) (Franceschini *et al.*, 2006; Smith *et al.*, 2006; Adachi *et al.*, 2007; Clarkson *et al.*, 2008). Recent observations suggest that sex steroids regulate the positive and negative actions on different subpopulations of hypothalamic kisspeptin neurones (Figure 1.7). During proestrous in rodents (or late in follicular phase of the menstrual cycle in primates), rising plasma oestradiol levels triggers a surge of GnRH-1 and LH secretion which induces ovulation. In rodents, this positive feedback effect of oestradiol appears to involve kisspeptin neurones in the AVPV, which act directly on GnRH-1 neurones to stimulate the preovulatory surge of GnRH-1 and LH (Wintermantel *et al.*, 2006). Studies have shown than the synchronisation of GnRH-1 neurones are facilitated by dendro-dendritic bundling and shared synapses between these neurones (Campbell *et al.*, 2009b). In the mouse, the expression of *Kiss1* mRNA in the AVPV is dramatically induced by oestradiol (Smith *et al.*, 2005a; Dungan *et al.*, 2007). Furthermore, in the rat, the expression of *Kiss1* mRNA in the AVPV peaks at a time coincident with the GnRH-1/LH surge and kisspeptin neurones in the AVPV show Fos induction at precisely this time (Smith *et al.*, 2006; Adachi *et al.*, 2007). Finally, mice bearing targeted deletions in *Gpr54* lack a LH surge (Clarkson *et al.*, 2008).

Recent studies suggest that kisspeptin-GPR54 signalling in the Arc mediates the negative feedback action of sex steroids on GnRH-1/LH secretion. After castration in mice, rats, hamsters and monkeys, levels of *Kiss1* mRNA increase dramatically in the Arc and this negative feedback effect can be reversed with sex steroid replacement (Irwig *et al.*, 2004; Navarro *et al.*, 2004; Smith *et al.*, 2005b; Revel *et al.*, 2006; Shibata *et al.*, 2007). Kisspeptin neurones in the Arc of the mouse express androgen receptor (AR) and ERα, suggesting that they are direct targets for the action of sex steroids (Smith *et al.*, 2005b). Kisspeptin neurones appear to play a key role in the negative feedback action of oestradiol in females as they do for testosterone in males. Ovariectomy caused an increase in *Kiss1* mRNA in the Arc of rodents, sheep and monkeys, and this increase is reversible upon treatment with oestradiol (Navarro *et al.*, 2004; Smith *et al.*, 2005a; Dungan *et al.*, 2007; Rometo *et al.*, 2007; Smith *et al.*, 2007; Smith *et al.*, 2008).

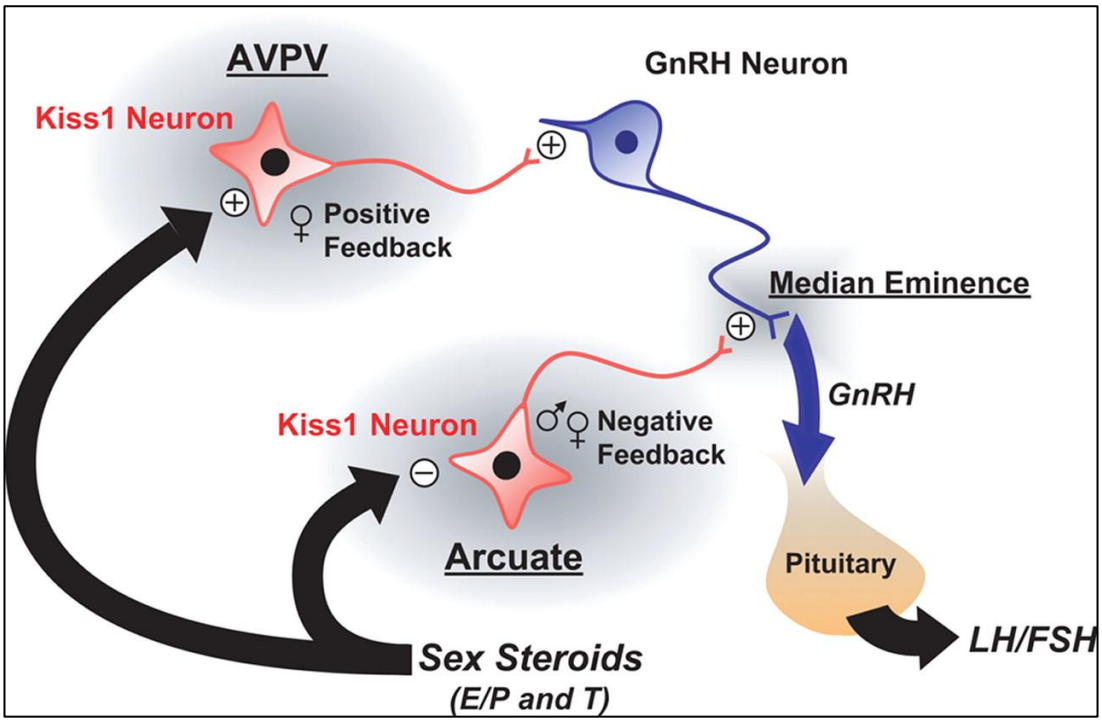


Figure 1.7: A schematic representation of kisspeptin signalling in the forebrain of the mouse. Kisspeptin stimulates GnRH secretion by a direct effect on GnRH neurones via GPR54. Kisspeptin neurones reside in the AVPV and Arc. Kisspeptin neurones in the Arc appear to be involved in the negative feedback regulation of GnRH/LH by sex steroids. These same sex hormones induce *Kiss1* mRNA expression in the AVPV, where kisspeptin neurones are thought to be involved in the positive feedback regulation of GnRH/LH. Modified from Gottsch *et al.*, 2006.

- **Sexual differentiation of the kisspeptin system**

Sexual differentiation of the kisspeptin neural circuitry is species specific. In adult sheep, the Arc is sexually differentiated, with ewes expressing higher numbers of kisspeptin neurones than rams (Caraty *et al.*, 1998; Estrada *et al.*, 2006). This finding is not so surprising given the putative role of ovine Arc in mediating the sexually dimorphic GnRH-1/LH surge. In the rodent, the AVPV is sexually differentiated, being larger and comprising more cells in the female than the male (Forger, 2009; Semaan *et al.*, 2010). This reflects the sexual differentiation of many neuronal phenotypes, including tyrosine hydroxylase (TH) neurones, neurotensin neurones and kisspeptin neurones (Clarkson and Herbison, 2006; Adachi *et al.*, 2007; Kauffman *et al.*, 2007). Since the AVPV is thought to play a critical role in relaying the positive feedback effects of oestradiol to GnRH-1 neurones, this may explain why the male rodent is incapable of generating a GnRH-1/LH surge (Smith *et al.*, 2006; Dungan *et al.*, 2007). The sex difference in the kisspeptin neural circuitry of the adult rodent is organised perinatally, as evidenced by the fact that neonatally androgenised females display a male-like

pattern of *Kiss1* expression in the AVPV and fail to generate a GnRH-1/LH surge in adulthood (Kauffman *et al.*, 2007; Navarro *et al.*, 2009). Likewise, neonatally castrated males show a feminised pattern of *Kiss1* expression in the AVPV (Homma *et al.*, 2009). However, *Kiss1* expression in the Arc of adult rodents is not sexually differentiated (Kauffman *et al.*, 2007).

- **Reproductive suppression in mole-rats**

In social mole-rats, reproductive control ranges from infanticide of the offspring of a subordinate, aggression and interference with mating attempts, to the suppression of the reproductive physiology of other members in the colony (Faulkes and Abbott, 1997). In eusocial mole-rats species (naked and Damaraland mole-rats), breeding opportunities are monopolised by dominant animals; usually a single female and one or two males that are responsible for procreation in a colony (Jarvis and Bennett, 1993). In mole-rats, the extent and type of reproductive suppression is correlated with the degree of sociality (Figure 1.3). Reproductive suppression amongst subordinate animals can be behavioural or physiological, or even a combination of the two. Behavioural suppression entails interference with the breeding attempts of subordinate animals by dominant animals. In the extreme, reproduction can be completely suppressed by blocking ovulation (Faulkes *et al.*, 1990a). The naked mole-rat is placed at the apex of this continuum with the most stringent level of reproductive suppression exerted by dominant animals (Figure 1.3). This unequal distribution of reproduction creates a reproductive skew, which differs significantly between species.

A number of models have been proposed to explain reproductive skew in cooperatively breeding societies. Firstly, the dominant control model suggests that the dominant members of the naked and Damaraland mole-rat colonies (known as the 'queen') exert some form of reproductive control over subordinate non-breeding females and males (Faulkes and Bennett, 2001). If this is true, it is still not known how the dominant queen achieves this. Previous studies have failed to reveal a role of urinary pheromones in the inhibition of reproductive activity in subordinate naked mole-rats, although it remains possible that volatile chemicals that are not present in urine could be involved in reproductive suppression (Faulkes and Abbott, 1993; Smith *et al.*, 1997). Alternatively, it has been suggested that the queen inhibits reproduction by behavioural intimidation, specifically by the repeated prodding and pushing of subordinate non-breeders (Reeve, 1992).

Secondly, reproductive skew may be explained by a self-restraint model specifically, incest avoidance. Most cooperatively breeding mammals live in extended family groups, and because breeding with close relatives is often deleterious, most species have evolved mechanisms to prevent inbreeding (Cooney and Bennett, 2000).

In colonies of Damaraland mole-rats, a combination of dominant control by the queen and incest avoidance may prevent reproduction among subordinates. Damaraland mole-rats are obligate outbreeders and will generally prefer mating with unfamiliar males than related males and foreign males are introduced into a colony (Cooney and Bennett, 2000; Burland *et al.*, 2004).

Reproductive female naked mole-rats are spontaneous ovulators and have an ovarian cycle of approximately 34 days (Faulkes *et al.*, 1990a). For reproductive naked mole-rats, a copulatory episode consists of the breeding females 'backing' up to a male in a tunnel while exhibiting a lordosis-like posture, following by the male mounting the female and pedalling his hind legs in an effort to establish genital contact (Jarvis, 1981). Copulation occurs when the male and female genitals contact each other and pelvic thrusting by the male is observed. The reproductive suppression of subordinate female and male naked mole-rats is profound. Subordinate females are anovulatory, have undetectable levels of urinary and plasma progesterone, low plasma LH levels, and a reduced pituitary LH response to exogenous GnRH-1 injections (Faulkes *et al.*, 1990a; Faulkes *et al.*, 1990b; Jarvis, 1991). When removed from the queen and paired with a male, subordinate females show an increase in urinary progesterone to a level commonly observed in breeding females (Faulkes *et al.*, 1990a).

Damaraland mole-rats are thought to be spontaneous ovulators, but the act of coitus may advance the onset of ovulation (Snyman *et al.*, 2006). In the Damaraland mole-rat, physiological suppression of reproduction is apparent in subordinate females. Female subordinates have small uteri, are anovulatory and remain in a pre-pubertal state throughout their life (Bennett and Jarvis, 1988). Reproductive female Damaraland mole-rats are characterised by their prominent teats and perforate vaginas. Subordinate female Damaraland mole-rats have low plasma and urinary progesterone levels, and low levels of urinary oestradiol compared to breeding female Damaraland mole-rats (Bennett and Jarvis, 1988; Bennett, 1994). Both subordinate and reproductive Damaraland mole-rats are able to produce an LH surge from the pituitary in response to a GnRH-1 challenge, however, reproductive females show a greater LH response at lower doses of GnRH-1 compared to subordinates (Bennett *et al.*, 1993). Furthermore, plasma progesterone concentrations rise when subordinate female Damaralands are separated from the queen. Indeed, after ovariectomy, reproductively-activated females continued to show significantly greater GnRH-1-stimulated LH secretion than subordinate females, indicating some independence from gonadal steroids (Molteno and Bennett, 2000).

Reproductive male Damaraland mole-rats are distinguished from subordinate females by their bulging testes, however, unlike naked mole-rats, only female Damaraland mole-rats have a physiologically suppressed reproductive system (Bennett

et al., 1996). In contrast to females, there are no differences in the physiology or endocrinology between subordinate and reproductive males. In male Damaralands, no differences in urinary testosterone, plasma LH or the LH response to a GnRH-1 challenge were found between subordinate and reproductive males (Bennett *et al.*, 1993; Bennett, 1994). Testis weights were greater in reproductive males compared to subordinates, however, no differences in the numbers of spermatozoa were detected (Faulkes *et al.*, 1994). This suggests that reproductive inhibition in subordinate male Damaraland mole-rats may be strictly behavioural and independent from circulating hormones. Thus, the reproductive suppression of male subordinate Damaraland mole-rats is behavioural only.

- **Sexual differentiation in mole-rats**

Sexual dimorphisms have been reported in the nervous system in all vertebrates and are thought to underlie sex differences in reproductive physiology and behaviour (Morris *et al.*, 2004). Sex differences in the nervous system are found in the spinal nucleus of the bulbocavernosus (SNB) (Breedlove and Arnold, 1980), the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Gorski *et al.*, 1978) and arginine vasopressin (AVP) innervations of the brain of rats (De Vries and Panzica, 2006). The SNB (also called Onuf's nucleus) motoneurones are larger and more numerous in male rodents and the target muscles are absent or vestigial in females (Breedlove and Arnold, 1980). The volume of the SDN-POA is dimorphic and is larger in males than female rats (Gorski *et al.*, 1978). Also, the lateral septum (LS) has a higher density of AVP innervations in males than females (De Vries and Panzica, 2006). Sex differences in the nervous system have evolved as specialisations that support the different reproductive strategies of the sexes. Males typically mount, intromit and ejaculate while females exhibit receptive behaviours, thus both sexes have evolved the morphological and neural adaptations that facilitate their respective behaviours (Morris *et al.*, 2004).

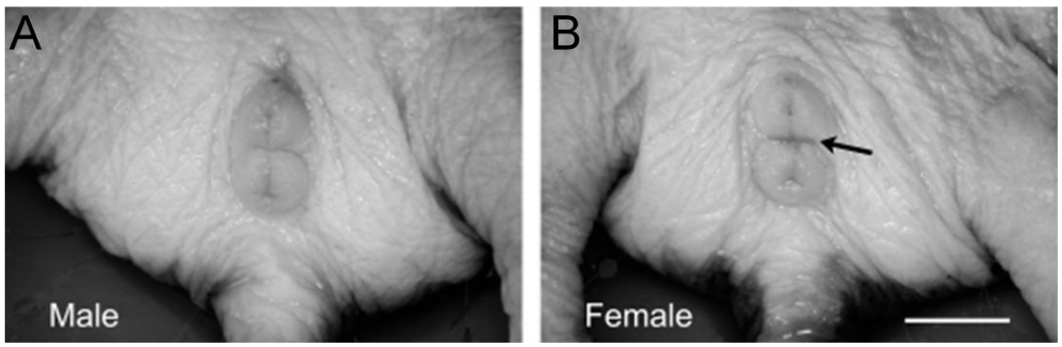


Plate 1.1: External genitalia of a (A) male and a (B) female subordinate naked mole-rat. The genital mound is anterior to the anal mound and has a very similar appearance in both sexes.

The vagina is not perforate in subordinate females; the arrow denotes where the vagina opens in breeders. Scale bar= 5mm. Modified from Peroulakis *et al.*, 2002.

For many aspects of their morphology, behaviour and neurobiology, naked mole-rats are remarkably monomorphic. There are no sex differences in overall body size or weight (Peroulakis *et al.*, 2002), and the external genitals are almost identical in subordinate males and female naked mole-rats (Plate 1.1). The penis or clitoris is immediately adjacent to the anus in both sexes and ano-genital distance does not differ by sex (Peroulakis *et al.*, 2002). All males are non-scrotal, the external penis is tiny and virtually identical to the clitoris (or female genital mound). Also, the vagina is imperforate in subordinate female naked mole-rats (Peroulakis *et al.*, 2002). There are also no sex differences for many of the behaviours displayed by subordinate naked mole-rats. Subordinate males and females participate equally in all sub-categories of grooming, resting, feeding, thermoregulation, locomotion, coprophagy, transport of food, digging, neonate tending and alarm reactions (Lacey and Sherman, 1991). In subordinate naked mole-rats, all features of the Onuf's nucleus (including the perineal muscle morphology, motoneurone number and size) are sexually monomorphic (Peroulakis *et al.*, 2002). Furthermore, there are also no sex differences in the perineal muscles and motoneurones within breeding naked mole-rats. Within both breeders and subordinates, there are no sex differences in overall volume, cell number or cell size in the ventromedial hypothalamic nucleus (VMH), BNST, paraventricular hypothalamic nucleus (PVH) or MeA (Holmes *et al.*, 2007). Thus, in many aspects of their morphology, behaviour and neurobiology, both the subordinate and reproductive naked mole-rats appear to be sexually monomorphic within their respective social classes.

Whilst naked mole-rats display a lack of sexual differentiation within their respective social classes, there are remarkable changes to their body morphology, behaviour and neurobiology when they become reproductively-activated (Peroulakis *et al.*, 2002; Seney *et al.*, 2006; Holmes *et al.*, 2007; Holmes *et al.*, 2008; Seney *et al.*, 2009). It thought that sexual differentiation might be paused in subordinate naked mole-rats, only emerging when subordinates become reproductively-activated. Female breeding naked mole-rats (the Queen) are morphologically different to subordinates. Breeding female naked mole-rats display an increase in body mass and develop an elongated body associated with an actual lengthening of vertebrae (O'Riain *et al.*, 2000; Dengler-Crish and Catania, 2007). This lengthening is thought to occur during successive pregnancies, and may represent a morphological adaptation that allows the breeding female to maneuver through narrow tunnels during the gestation of large litters. Also, breeding naked mole-rats of both sexes have approximately 30% more

Onuf's nucleus motoneurone recruitment than subordinates (Seney *et al.*, 2006). Reproductive females display a lordosis-like posture and reproductive males display reproductive copulatory behaviours, such as, ano-genital nuzzling, mounting of the female and pelvic thrusting− behaviours which are not observed in subordinate naked mole-rats (Jarvis, 1981).

Reproductive naked mole-rats also show significant differences in their neurobiology in comparison to subordinates. Regardless of sex, breeders have more neurones in the VMH and larger volumes for the BNST, PVH and MeA than subordinates− regions which have been associated with reproductive functions and sexual dimorphism in other rodents (Holmes *et al.*, 2007). Moreover, a history of breeding is not a prerequisite for such changes, simply pairing a male and female subordinate in isolation from the queen is sufficient to cause breeder-like changes in the PVH (Holmes *et al.*, 2011). These changes appear to be independent of changes in circulating levels of gonadal steroids since long-term gonadectomy does not reverse the breeder-like neural changes in the PVH or BNST (Holmes *et al.*, 2011). Thus, the neural changes associated with the transition from subordinate breeding status remain largely independent from gonadal hormones. Taken together, these data indicate that social status is a more important factor than sex for determining neural morphology in naked mole-rats.

Like naked mole-rats, breeding Damaraland mole-rats have larger volumes of the BNST and PVH than do subordinates, with no effect of sex on these measures (Anyan *et al.*, 2011). However, in contrast to naked mole-rats, the volume of the MeA and the motoneurone number in Onuf's nucleus are both greater in male than in female Damaraland mole-rats, with no significant effect of breeding status (Anyan *et al.*, 2011). Thus, both sex and breeding status influence neural morphology in Damaraland mole-rats. These findings are in accord with the observed sex differences in body weight and genitalia in Damaraland but not naked mole-rats (Anyan *et al.*, 2011). So despite being eusocial, the increased sexual dimorphism in Damaraland mole-rats relative to naked mole-rats may be related to their reduced reproductive skew.

In a comparison of the genitalia and perineal muscles in three different mole-rat species with varying social structures: naked mole-rats lack sex differences, silvery mole-rats (*Heliophobius argenteocinereus*) exhibit sex differences, and Damaraland mole-rats are intermediate, specifically, the external genitalia and one of the three perineal muscle are sexually dimorphic in Damaraland mole-rats (Seney *et al.*, 2009). The naked mole-rat is eusocial, the silvery mole-rat is a strictly solitary species and the Damaraland mole-rat is eusocial, but has a smaller colony size and has less reproductive skew than naked mole-rats. In this sense, Damaraland mole-rats may be considered intermediate in social organisation between naked mole-rats and silvery

mole-rats (Seney *et al.*, 2009). These findings support a relationship between social structure and degree of sexual differentiation, whereby eusocial mole-rats are sexually monomorphic and solitary mole-rats are sexually differentiated.

- **The neurobiology of social behaviour**

Many types of social interactions exist between individuals at both an intra-specific and to a lesser degree inter-specific level in the animal kingdom. Intra-specific behaviours include acts, such as mating, aggression and maternal care, which are ubiquitous amongst all mammalian species. However, the formation of strong bonds between mating pairs (pair bonding) are far more unusual. Monogamy is rare in mammals; less than 5% of all mammals form a pair bond during their reproductive periods (Kleiman, 1977). Monogamy comprises a set of complex behavioural components, including pair bonding in males and females, parental care, and increased aggression in the context of home range defence (Kleiman, 1977; Keverne and Curley, 2004). It is thought that the rarity of monogamy is due to the contrasting reproductive strategies of males and females. Reproductive success in males is normally determined through competition with other males to mate with as many females as possible, whereas reproductive success in females is dependent on the production of a smaller number of healthy offspring (Keverne and Curley, 2004).

Neuropeptides of the nine amino acid arginine vasotocin (AVT) family, which include the mammalian peptides arginine vasopressin (AVP) and oxytocin (OT), have evolutionarily conserved structures and functions in vertebrate animals (Goodson and Bass, 2001). Mammalian social bonds are underpinned by the hormones OT and AVP. These closely related peptides are produced by magnocellular neurones in the PVH and supraoptic nucleus (SON) of the hypothalamus and are released into the bloodstream at the posterior pituitary (Bielsky and Young, 2004). In addition, extra-hypothalamically produced AVP (specifically in the BNST, MeA and SCN) and OT (specifically the parvocellular neurones of the PVH) are released in specific regions throughout the rodent forebrain; and it is thought that these centrally–projecting neurones modulate social functions (De Vries and Buijs, 1983). Furthermore, OT and AVP are also crucial for the regulation of other aspects of mammalian social behaviour, such as aggression, anxiety and male territorial marking (Le Moal *et al.*, 1987; Landgraf *et al.*, 1995; Everts and Koolhaas, 1997). For example, vasopressin receptor (V1aR) KO mice exhibit markedly reduced anxiety behaviour and impaired social olfactory recognition (Bielsky *et al.*, 2004).

In mammals, social recognition memory is critically important in the context of mate and offspring recognition, and is dependent upon olfactory cues. The brain's oxytocinergic system together with olfactory recognition memory underlies the

formation of female social bonds with mates or offspring, and is brought about by prolonged contact with unfamiliar males or newly born offspring (Keverne and Curley, 2004). The formation of a familiar relationship in males involves sexual activity, whereas offspring recognition immediately follows parturition. During pregnancy, the hormones progesterone and oestrogen prime the brain for maternity by inducing the synthesis of OT receptors (OTR) in the central olfactory projections (olfactory bulb (OB), MeA and POA), as well as OT and dopamine receptors in the NAcc− the area of the brain's dopaminergic reward pathway (Liu and Wang, 2003). Furthermore, OT release at parturition regulates maternal behaviour and physiology, such as, uterine contractions and milk ejection (Pedersen and Prange, 1979). In sheep, OT acting in the OB is involved in the long-term olfactory recognition memory of a newborn lamb (Keverne and Kendrick, 1992).

Oestrogen acts through the steroid nuclear receptors ERα and ERβ, which regulate gene transcription (Mitra *et al.*, 2003). ERα-, ERβ- and OT KO mice all fail to display social recognition, despite normal olfactory and spatial learning abilities; indicating that the genes for both ERα and ERβ play a crucial role in OT-dependent social olfactory recognition (Ferguson *et al.*, 2000; Choleris *et al.*, 2003). Furthermore, treatment with OT in the MeA rescued social memory in OT KO mice and treatment with an OT antagonist produced a social amnesia-like effect in wild-type mice (Ferguson *et al.*, 2000). Using c-Fos immunoreactivity as a marker of neuronal activation, only wild-type mice (and not OT KO mice) showed an induction of Fos-immunoreactivity in the MeA (Ferguson *et al.*, 2001).

Other researchers have proposed that (1) oestrogens (through ERα and ERβ) regulate OT secretion in the PVH and SON, and via axonal projections from the hypothalamus to the MeA, and (2) oestrogen mediated OT-OTR activation in the MeA ultimately facilitates social olfactory recognition in rodents (Choleris *et al.*, 2003). While many of the roles of OT are associated with female-typical behaviours, most of the behaviours associated with AVP (e.g. aggression) have been demonstrated in males (Delville *et al.*, 1996; Ferris *et al.*, 1997). Moreover, AVP modulates social behaviour in a species-specific manner. For example, AVP facilitates scent-marking behaviour in hamsters (Ferris *et al.*, 1984), aggression in hamsters (Ferris *et al.*, 1997) and plays a role in paternal care in male voles (Winslow *et al.*, 1993; Wang *et al.*, 1994).

Much of our current understanding of the neurobiology of affliative behaviour comes from studies on species with unique social structures. Voles of the genus *Microtus* offer an excellent model system to study the neurobiology of social behaviour. The monogamous prairie voles (*Microtus ochrogaster*) and pine voles (*Microtus pinetorum*) form pair bonds and show bi-parental care of offspring, whilst the promiscuous meadow voles (*Microtus pennsylvanicus*) and montane voles (*Microtus*

montanus) are solitary and show only maternal care of offspring (Williams *et al.*, 1992; Carter and Getz, 1993; Insel *et al.*, 1994). Female prairie voles are induced into oestrus by pheromones from the male and mating; this in turn stimulates central OT release which enables the females to form a partner-preference bond (Williams *et al.*, 1992). In the absence of mating, central administration of OT can also induce partner preferences in sexually naïve female prairie voles (Williams *et al.*, 1994)**.** Furthermore, if pre-treated with an OT antagonist into the medial prefrontal cortex (mPFC), this inhibited the formation of mating-induced partner preference in female prairie voles (Liu and Wang, 2003). In male prairie voles, mating leads to the central release of AVP along with the development of a pair-bond, increased aggression towards foreign males and parental care of young (Winslow *et al.*, 1993). More specifically, administration of V1aR antagonist directly into the LS or ventral pallidum (VP) of male prairie voles inhibited the formation of mating-induced partner preference (Liu *et al.*, 2001; Lim and Young, 2004).

- **The modulation of adult neurogenesis**

Neurogenesis is a process that consists of both cell proliferation and cell survival. New neurones continue to be generated in two areas of the adult brain: the dentate gyrus (DG) (Plate 1.2) of the hippocampus and the subventricular zone (SVZ). Neurones born in the SVZ migrate through the rostral migratory stream RMS) where they differentiate into interneurones in the OB (Peretto *et al.*, 1999). Progenitor cells born in the subgranular zone (SGZ) of the DG proliferate and migrate into the granule cell layer (GCL), becoming granule cells of the DG. Later, these granule cells extend their dendrites toward the molecular layer (ML) of the DG (Cameron *et al.*, 1993; von Bohlen und Halbach, 2011). These newly formed neurones are then incorporated into functional networks of the DG and SVZ, leading to a significant impact on cognitive functions. More recent studies have documented newly proliferated neurones in other brain regions, including the amygdala and hypothalamus (Fowler *et al.*, 2002; Fowler *et al.*, 2003; Fowler *et al.*, 2005).

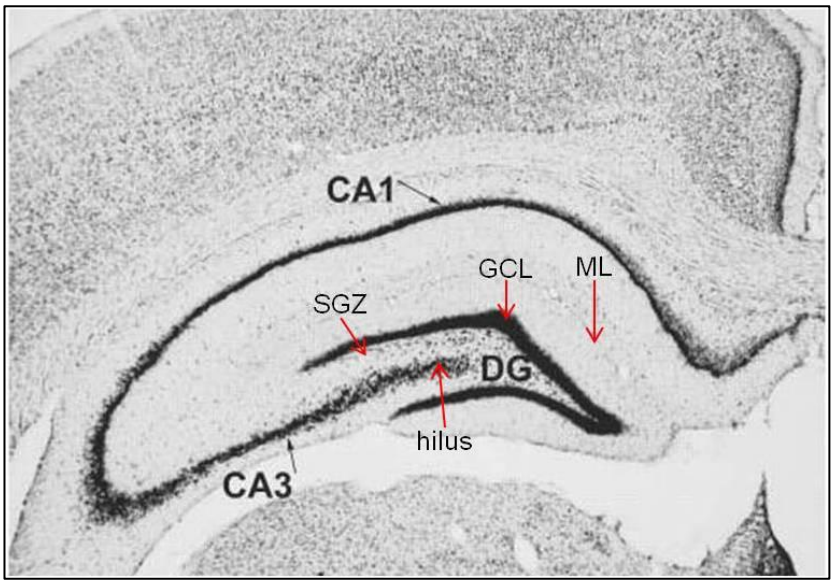


Plate 1.2: Nissl-stained coronal section of the hippocampus. CA1 and CA3 pyramidal cell layers of the hippocampus; DG, dentate gyrus; GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone. Progenitor cells are born SGZ of the DG, then proliferate and migrate into the GCL, then finally extend their dendrites toward the ML. Modified from Romeo *et al.*, 2004.

Although neurogenesis occurs continuously throughout adulthood, the rate of proliferation may be affected by many factors, such as, pubertal development (Ho *et al.*, 2011), environmental enrichment (Kempermann *et al.*, 1997,1998; Nilsson *et al.*, 1999), photoperiod (Huang *et al.*, 1998), breeding season (Galea and McEwen, 1999), psychosocial stress (Kozorovitskiy and Gould, 2004; Thomas *et al.*, 2007), care of young (Shingo *et al.*, 2003; Ruscio *et al.*, 2008) and exposure to conspecifics (Smith *et al.*, 2001; Fowler *et al.*, 2002). The exposure of female prairie voles to males is associated with neurogenesis in the amygdala, hypothalamus and SVZ of females (Fowler *et al.*, 2002). Female prairie voles induced into oestrus by male exposure have increased number of 5-bromo-2'-deoxyuridine- (BrdU) labelled cells (a marker of proliferating cells) in the SVZ and RMS (Smith *et al.*, 2001). Ovariectomised females exposed to males did not show an increase in number of BrdU-labelled cells in the SVZ, indicating the role of oestrogen in this phenomenon (Smith *et al.*, 2001). In addition to the effects of pair-bonding and mating, exposure to neonate pups also elicits hippocampal cell proliferation in the prairie vole (Ruscio *et al.*, 2008). Neurogenesis in the SVZ has also been implicated in the development of maternal behaviour in mice and can be induced by prolactin (Shingo *et al.*, 2003). These pregnancy-stimulated neuronal progenitors migrate to produce new olfactory interneurones, crucial for the development of maternal behaviour, since olfactory discrimination is critical for offspring recognition.

A long-standing association between stress and neurogenesis has been supported by many laboratory studies on depression (Fuchs and Flugge, 1998; Gould

and Tanapat, 1999). Studies have shown that elevated glucocorticoid levels during times of stress leads to reduced growth of new neurons in the DG of the hippocampus (Cameron and Gould, 1994; Tanapat *et al.*, 1998; Mirescu *et al.*, 2006). Psychosocial stress caused by dominance hierarchy formation (i.e. a pattern of repeated agonistic interactions with an expected outcome of winner and loser) has been shown to influence neurogenesis in the hippocampus (Kozorovitskiy and Gould, 2004; Pravosudov and Omanska, 2005). Rats exposed to a social dominance paradigm (an acute psychosocial stressor that leads to behavioural and physiological responses) at the time of cell generation resulted in a decreased number of newly generated cells in the hippocampus (Thomas *et al.*, 2007). In a similar study, more new neurones were observed in the DG of the more dominant male rats compared to the more subordinate rats, and that social dominance increases the number of new neurones by enhancing cell survival rather than cell proliferation (Kozorovitskiy and Gould, 2004).

Gonadal hormones have powerful effects on both brain structure and behaviour, throughout the life of an animal. Repeated oestradiol exposure up-regulates cell proliferation and suppresses apoptosis in the females but not male rat (Barker and Galea, 2008). Oestradiol treatment enhances hippocampal neurogenesis and decreases cell death in the DG of adult female rats (Tanapat *et al.*, 1999). Testosterone and dihydrotestosterone are found to enhance survival of new hippocampal neurones in adult male rats (Spritzer and Galea, 2007). Gonadal hormones modulate neurogenesis in the DG differentially in male and female adult rodents. After exposure to acute or chronic stress, adult male rats show reduced neurogenesis in the DG, whereas the females show no significant change (Falconer and Galea, 2003). Suggesting that there are sex differences in the response of hippocampal neurogenesis not only to gonadal hormones, but also to stress hormones.

- **Study animals:**
  - **The naked mole-rat (*Heterocephalus glaber*) (Plate 1.3)**

Naked mole-rats inhabit large subterranean burrow systems in the semi-arid regions of East Africa (Jarvis, 1981; Brett, 1991). They live in a network of burrows and chambers excavated out of sandy soil, which can reach lengths of over 3km depending on colony size and food availability (Bennett and Faulkes, 2000a,b). This network consists of foraging ‘galleries’, deeper more permanent ‘highways’, and a multi-entranced nest chamber, food store and toilet chambers (Brett, 1991). Naked mole-rats have long gestation period of approximately 70 days and an average litter size of 11 (Roellig *et al.*, 2011). The reproductive female has an ovarian cycle of approximately 34 days (Faulkes *et al.*, 1990a). Naked mole-rats are spontaneous ovulators (Faulkes *et al.*, 1990a) and can live for up to 30 years (Buffenstein, 2008). These eusocial rodents live

in large colonies of 70-80 and practice an extreme form of cooperative breeding, in which reproduction is restricted to one dominant female, the queen, and one to three males (Jarvis, 1981,1991).

Plate 1.3: The naked mole-rat (*Heterocephalus glaber*). Taken by Steve Gorton.

In response to the presence of the queen, the rest of the subordinate colony members show suppressed reproductive physiology and behaviour; they perform supporting roles, such as burrowing, foraging, care for offspring and defending the colony (Faulkes *et al.*, 1990a; Faulkes and Abbott, 1991; Faulkes *et al.*, 1991; Lacey and Sherman, 1991). Parturition and nursing are exclusive to the queen while other aspects of pup care, such as carrying and grooming, are shared among all colony members (Lacey and Sherman, 1991). Genital nuzzling behaviour is frequently performed by the breeders, and is almost never performed by subordinate members of naked mole-rat colonies. Genital nuzzling occurs at all times of the female's ovulatory cycle and during pregnancy and continues to be expressed even after gonadectomy of the breeding pair (Goldman *et al.*, 2006). When removed from the queen, subordinate naked mole-rats can become reproductively-activated (Faulkes and Abbott, 1991,1997). Naked mole-rats differ from other mole-rat species in that they will spontaneously inbreed with no incest avoidance, although when given a choice, they prefer outbreeding (Clarke and Faulkes, 1999; O'Riain *et al.*, 1996).Consequently, there is a very high reproductive skew among members of their colonies and lifetime reproductive success is almost zero (Jarvis *et al.*, 1994).

Following the death or removal of a breeder, subordinate naked mole-rats may achieve breeding status within their natal colony. Loss of the queen is often followed by fighting among a subset of subordinate females before a new queen becomes established (Clarke and Faulkes, 1997). Once a queen is established, she becomes morphologically distinct from subordinates. Breeding female naked mole-rats develop an elongated body associated with actual lengthening of the vertebrae (O'Riain *et al.*, 2000). Most of the vertebral lengthening occurs during successive pregnancies and

may represent an adaptation that allows the breeding female to manoeuvre through narrow tunnels during gestation of large litters (Bennett and Faulkes, 2000b).

The breeding females (the queen) is usually at the top of the colonial hierarchy and in rare succession events she is likely to be replaced by the next highest ranking female (Clarke and Faulkes, 1997). A significant negative correlation between body mass/age/urinary testosterone levels and dominance rank has been identified (Clarke and Faulkes, 1997). Therefore, the most dominant animals are usually the largest, oldest and have the highest testosterone levels. However, other studies have shown that dominance hierarchy is based on body weight alone, discarding age as a variant (Jarvis, 1991). Previous studies have used the outcomes of passing behaviours to determine dominance hierarchy within a colony. Passing behaviour is an asymmetrical interaction between two naked mole-rats encountering each other face-to-face in a tunnel. After a brief period of facial sniffing (which implies recognition), the more dominant individual passes over the top of the other (Clarke and Faulkes, 1997).

There may also be a 'disperser morph' in naked mole-rats, these disperser males have large deposits of subcutaneous adipose tissue in the neck region (possibly to provide energy reserves during dispersal) (O'Riain *et al.*, 1996). Disperser naked mole-rat males are laden with fat, exhibit elevated levels of LH, have a strong urge to disperse after periods of rainfall when the soil is soft, and only solicit matings with non-colony members (O'Riain *et al.*, 1996).

- **The Damaraland mole-rat (*Fukomys damarensis*)**

Damaraland mole-rats live in colonies of up to 41 individuals and exhibit an extreme form of socially induced infertility among mammals (Bennett *et al.*, 1993). They have a long gestation period of approximately 78-92 days and an average litter size of 3 (Bennett and Jarvis, 1988; Jarvis and Bennett, 1993). Damaraland mole-rats have smaller colony sizes and have a smaller reproductive skew than naked mole-rats. Damaraland mole-rats are obligate outbreeders and will generally prefer mating with unfamiliar mates than related males and foreign males are introduced into a colony (Cooney and Bennett, 2000; Burland *et al.*, 2004). They are spontaneous ovulators (Snyman *et al.*, 2006) and can live to at least 15 years in the laboratory (Holmes *et al.*, 2009).

Plate 1.4: The Damaraland mole-rat (*Fukomys damarensis*). Taken by Tim Jackson.

Within the colony, only one dominant female (the queen) and male (occasionally two) are capable of breeding, and the vast majority of individuals will remain subordinate (Bennett, 1990). Subordinate Damaraland mole-rats fall into the two categories (frequent and infrequent workers), where the former look after the young in the colony and the latter may act as dispersers who build up their own body reserves in preparation for dispersal when environmental conditions are suitable (Scantlebury *et al.*, 2006). However, unlike naked mole-rats, only female Damaraland mole-rats have a physiologically suppressed reproductive system (Bennett *et al.*, 1996). In contrast to females, there are no differences in the physiology or endocrinology between subordinate and reproductive males (Bennett, 1994). Thus, reproductive inhibition in subordinate male Damaraland mole-rats may be strictly behavioural and independent from circulating hormones.

AIMS

The eusocial naked and Damaraland mole-rats were the subjects of investigation in this thesis with the explicit purpose of increasing our knowledge on their neurobiology. Eusocial mole-rats are a unique animal model in which to study the effects of social and reproductive status on the plasticity of the mammalian nervous system. Both the naked and Damaraland mole-rats exhibit an extreme form of cooperative breeding in which only one dominant female (and 1-3 males) are capable of reproducing. Breeders of both sexes are socially dominant over subordinates of the colony and the dominant female can reproductively suppress the other subordinate members (Holmes *et al.*, 2009). In the laboratory, subordinate naked and Damaraland mole-rats can be reproductively-activated by removing them from their natal colonies and pairing them with opposite sex mates. I took advantage of this variability in social and reproductive status to address several questions related to the neurobiological basis of reproductive suppression, sociality and neurogenesis in these animals.

Reproductive suppression

The first objective of this thesis was to analysis the HPG system of the eusocial naked and Damaralad mole-rats. In chapter 3, the GnRH-1 and kisspeptin systems in reproductive and subordinate naked mole-rats of both sexes were compared to elucidate their potential roles in HPG axis activation. The effects of breeding history, gonadectomy and sex were assessed on GnRH-1- and kisspeptin-immunoreactivity. Also, steroidal hormone levels were measured and compared between the reproductive and subordinate naked mole-rats. In chapter 4, RFamide-immunoreactivity was also assessed in reproductive and subordinate Damaraland mole-rats of both sexes. Finally, in chapter 5, TH-immunoreactivity in the diencephalon of female and male naked mole-rats were assessed to elucidate sexual monomorphism in naked mole-rat neurobiology.

Social behaviour

The second objective of this thesis was to analyse the neurobiology of eusocial Damaraland mole-rats. In chapter 6, the distribution of OT- and AVP-immunoreactivity and the telencaphalic distribution of their receptors OTR and V1aR in the eusocial Damaraland mole-rat brain. In addition, this study addresses the comparisons in the OT and AVP and their receptor systems between different species of mole-rats, and between Old World mole-rats and New World voles.

Neurogenesis

The third objective of this thesis was to analyse BrdU- (cellular proliferation marker) and DCX- (immature neurone marker) immunoreactivity in the hippocampal dentate gyrus of reproductively-activated and subordinate naked mole-rats. In chapter 7, the rate of neurogenesis and the effects of reproductive status on neurogenesis was investigated in naked mole-rats.

This conclusion chapter synthesises the findings of this study and places them into the broader context in the regulation of reproduction, sociality and neurogenesis in African mole-rats and other mammalian species.

CHAPTER 2:

Materials

STUDY ANIMALS

- **Naked mole-rats**

Naked mole-rat colonies were maintained at the University of Connecticut, Storrs, USA and Queen Mary University of London, London, UK (the animals housed in London were studied for the work described in chapters 5 and 7). All animals in our colonies are identified by subcutaneous transponders (AVID Corporation, Norco, CA). In addition, animals' heads were marked with a marking pen for rapid identification during behavioural observations. Colonies were maintained in polypropylene tubs which contain bedding and were connected by lengths of acrylic tubing (Figure 2.1). Animals were fed *ad libitum* on sweet potato. Animals were housed at an ambient temperature of 30^{o}C (as this species has a very weak capacity for physiological thermoregulation and requires a high ambient temperature), under a 12:12 hour light/dark cycle. Naked mole-rats are very long-lived, can survive for over 30 years in captivity and reach adult body size between 4 and 24 months of age (Buffenstein, 2008). Animals in this study were between 2 and 17 years of age and weighed between 21.7 and 67.1g.

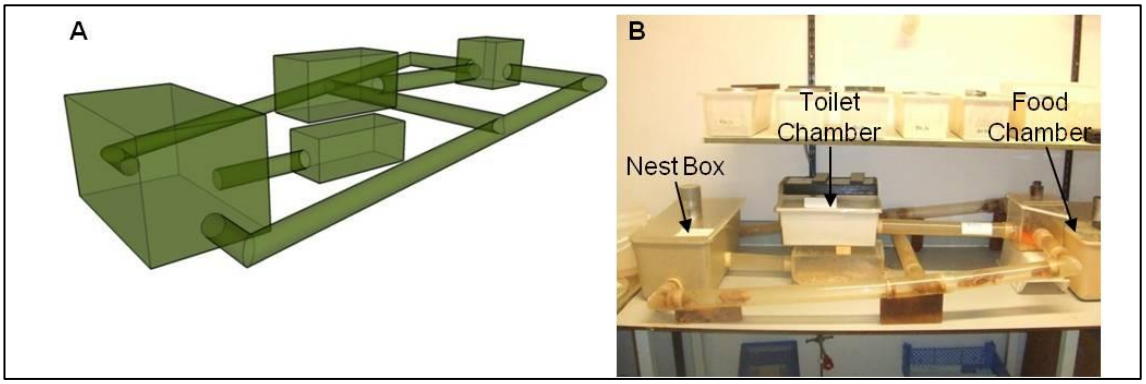


Figure 2.1: (A) Diagram of a naked mole-rat tunnel system, (B) labelled photograph of a naked mole-rat tunnel system containing nest box, toilet chamber and food chamber. Modified from Maciag, 2009.

Subordinates were animals that remained in their natal colonies until tissue collection. Subordinates were reproductively-activated by removing them from their colonies (at random) and housing them with an opposite sex individual from a different colony. Each individual was weighed regularly and assessed for signs of reproductive activation, such as, a perforate vagina (in females) and a display of sexual behaviours (ano-genital nuzzling). Importantly, the reproductively-activated animals had never produced a litter. The breeders were reproductively-activated female/male pairs that were separated from their natal colonies, paired in ‘mini’ non-functional colonies and

had produced at least one litter. They were classified as breeders once the female member of the breeding pair had given birth to at least one litter. Gonadectomised (GDX) breeders had been gonadectomised at least one year prior to collection. They had been paired for at least ten years prior to gonadectomy and had produced at least one litter.

Breeders, reproductively-activated animals and subordinates did not differ significantly in mean age, although GDX breeders were significantly older than all other groups ($p<0.05$). From a practical perspective, we were only able to create a limited number of female/male reproductively-activated pairs, and wait for them to give birth to at least one litter. As a result, it was difficult to obtain large numbers of breeding females in a specific reproductive phase. Due to the scarcity of animals, and the fact that established breeders are crucial to the continuation of our colonies, we were not able to study a group of queens, i.e. successful female breeders that had produced and raised young, were living in a functional colony with subordinate and were gonadally-intact.

All animal procedures were conducted according to USA institutional and federal guidelines, and were approved under the authority of a UK Home Office Animals (Scientific Procedures) Act 1986 and with regulations of the Queen Mary, University of London's Animal Ethics Committee.

- **Damaraland mole-rats**

Damaraland mole-rats were trapped at Hotazel, Northem Cape Province, South Africa ($27^{o}17'$; $22^{o}58'$ E). All animals were field-caught and brought back to the laboratory at the University of Pretoria and habituated for 2 months prior to experimentation. Colonies were housed in plastic crates with nesting boxes, wood-shaving and shredded paper towelling for nesting material. Individual colony members were identifiable by a unique, white occipital head patch. In order to facilitate rapid identification of individuals, different coloured vegetable dyes were applied to each animal's head patch. The animals were maintained at constant temperature ($25^{o}C$), in continuous darkness and were fed and cleaned under dim red light. Animals were provided with freshly chopped vegetables daily. Animals in this study weighed between 56g and 175g.

Subordinates were animals that remained in their natal colonies until tissue collection. Subordinates were reproductively-activated by removing them from their colonies (at random) and housing them with an opposite sex individual from a different colony. Each individual was weighed regularly and assessed for signs of reproductive activation, such as, a perforate vagina (in females) and bulging testes (in males). Importantly, the reproductively-activated animals had never produced a litter. The

breeders were reproductively-activated female/male pairs that were separated from their natal colonies, paired in ‘mini’ non-functional colonies and had produced at least one litter. They were classified as breeders once the female member of the breeding pair had given birth to at least one litter. GDX breeders had been gonadectomised at least four months prior to collection. Male breeders were significantly heavier than female breeders and subordinates of both sexes ($p < 0.05$).

From a practical perspective, we were only able to create a limited number of female/male reproductively-activated pairs, and wait for them to give birth to at least one litter. As a result, it was difficult to obtain large numbers of breeding females in a specific reproductive phase. Due to the scarcity of animals, and the fact that established breeders are crucial to the continuation of our colonies, we were not able to study a group of queens, i.e. successful female breeders that had produced and raised young, were living in a functional colony with subordinate and were gonadally-intact.

The animals were collected under permit from the Northern Cape Department of Nature Conservation.

TISSUE PREPARATION

- **Naked mole-rats**

Castrations were performed while animals were under avertin anaesthesia (30 mg/100 g) intraperitoneally (i.p.). For ovariectomies, animals were placed under isoflurane anaesthesia. Just prior to anaesthesia, a subcutaneous injection of analgesic (Meloxicam, 50 µg/100 g) was administered. The testes are retained in the abdomen in naked mole-rats, and abdominal incisions were required for their removal, as was the case for ovariectomies. Abdominal incisions and skin incisions were sutured with surgical thread following removal of the gonads. Animals were returned to their colonies within 4 hours following surgery and were accepted by the other members of the colony without incident.

All animals were weighed prior to sacrifice. For blood and brain collection, animals were anesthetised with avertin (40 mg/100 g) and rapidly decapitated. After decapitation, trunk blood was collected in heparinised tubes and placed on ice for a maximum of 1 hour. After collection, blood was centrifuged and the supernatant plasma was stored at -20^{o}C until plasma steroidal hormone analysis. The reproductive status of the animals was assessed by dissection and examination of the reproductive tracts. The adrenal glands were dissected and weighed. Brains were removed, fixed by immersion in 5% acrolein and 0.1 M phosphate buffered saline (PBS) (pH 7.4) for 4 hours, and sunk in 30% sucrose for cryoprotection. Brains were either quick frozen in dry ice and sectioned coronally at 30 µm into six sequential series on a cryostat (Bright Cryostats, UK), or brains were cut 30 µm thick sections on a freezing microtome into a series of four (for chapter 3 only). Brain slices were stored in cryoprotectant at -20°C until use. Sections from one series were mounted onto gelatine-subbed slides and stained with thionin to determine anatomical structures.

All animal procedures were conducted according to USA institutional and federal guidelines, and were approved under the authority of a UK Home Office Animals (Scientific Procedures) Act 1986 and with regulations of the Queen Mary, University of London's Animal Ethics Committee.

- **Damaraland mole-rats**

Castrations and ovariectomies were performed while animals were under inhalation of halothane. The testes are retained in the abdomen in Damaraland mole-rats, and abdominal incisions were required for their removal, as was the case for ovariectomies. Abdominal incisions and skin incisions were sutured with surgical thread following removal of the gonads. Animals were returned to their colonies within 4 hours following surgery and were accepted by the other members of the colony without incident.

All animals were weighed prior to sacrifice. For brain collection, Damaraland mole-rats were anaesthetised using halothane followed by intramuscular injection of 30 mg ketamine/kg and 5 mg rompun/kg. Individuals were perfused through the aorta with approximately 150ml 0.1 M PBS, followed by approximately 300 ml 4% paraformaldehyde (PFA) in 0.1 M PBS. Heads were removed and immersed in 4% PFA for 48 hours. Brains were removed from skulls and sunk in 30% sucrose overnight at 4^{o}C for cryoprotection. Brains were quick frozen in dry ice sectioned coronally at 30 µm into six sequential series on a cryostat (Bright Cryostats, UK) and stored at -20^{o}C in anti-freeze. All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and with the regulations of the University of Pretoria's Animal Ethics Committee.

CHAPTER 3:

Analysis of hypothalamic GnRH-1 and kisspeptin neuronal systems in reproductively-activated and subordinate naked mole-rats (*Heterocephalus glaber*)

ABSTRACT

Naked mole-rats (*Heterocephalus glaber*) live in large colonies in which breeding is monopolised by a dominant female (queen) and 1-3 male consorts. Subordinate colony members are reproductively suppressed by social cues from the queen and show no signs of hypothalamo-pituitary-gonadal (HPG) axis activation. However, fertility can be induced by removing subordinates from the queen. Kisspeptin, a potent gonadotrophin-releasing hormone (GnRH-1) secretagogue, is required for pubertal activation of the HPG axis in rodents and humans. This study investigated whether there are changes in the numbers of GnRH-1- and/or kisspeptin-immunoreactive (ir) cell bodies when adult subordinates are released from socially-induced reproductive suppression. These neurones were assessed in eight groups of animals: breeders, reproductively-activated, gonadectomised (GDX) breeders, and subordinates for both sexes. GnRH-1-ir cell bodies were distributed in a loose continuum along the septo-preoptico-hypothalamo pathway, with the vast majority of GnRH-1-ir cell bodies located in the medial septum (MS), the vertical (VDB) and horizontal diagonal bands of Broca (HDB), and the medial preoptic area (MPOA). Kisspeptin-ir cell bodies were identified at four sites: the rostral periventricular region of the third ventricle (RP3V), the paraventricular hypothalamic nucleus (PVH), the arcuate nucleus (Arc) and the dorsomedial hypothalamic nucleus (DMH). Kisspeptin-ir processes were present in the hypothalamus, the ventrolateral septum (VLS), the MS and nucleus accumbens (NAcc), but absent from the suprachiasmatic nucleus (SCN) and the ventromedial hypothalamic nucleus (VMH). There was no significant effect of reproductive status on the number of GnRH-1-ir cell bodies. Breeding/reproductively-activated animals had significantly more kisspeptin-ir cell bodies in the RP3V and PVH than subordinates. Furthermore, there was no effect of breeding history, gonadectomy or sex on the number of GnRH-1- or kisspeptin-ir cell bodies. These results indicate that the transition of naked mole-rats from subordinate to reproductive status (1) involves upregulation of the hypothalamic kisspeptin system and (2) may be relatively independent from gonadal hormones.

INTRODUCTION

A characteristic of cooperatively breeding mammals, where individuals other than the parents care for offspring, is that reproduction is limited to socially dominant individuals. For example, in some species of the South American primate family Callitrichidae, reproduction is monopolised by a single dominant female (Abbott *et al.*, 1998). Naked mole-rats offer a unique model through which the effects of social environment on HPG axis-activation can be studied in the laboratory. Naked mole-rats are an extreme example of not only cooperative breeding, but also of socially-induced infertility; in subordinates of both sexes, the physiological systems required for reproduction are suppressed by the queen (Jarvis, 1991). Moreover, several neuroanatomical and endocrine features vary with social status (Faulkes *et al.*, 1990a; Faulkes *et al.*, 1990b; Faulkes and Abbott, 1991; Faulkes *et al.*, 1991; Holmes *et al.*, 2011). So despite achieving adult body size, the endocrine system of subordinate naked mole-rats remains in a pre-pubertal state (see chapter 1 for general introduction on reproductive suppression in mole-rats).

Until recently, studies on the neuroendocrine control of reproduction in mammals have been limited to the hypothalamic GnRH-1 system. Kisspeptin, the RF-amide product of the *Kiss1* gene and the natural ligand of GPR54 (the kisspeptin receptor) has recently been found to be of major importance in a wide array of mammalian reproductive functions (see chapter 1). When a subordinate naked mole-rat is released from socially-induced reproductive suppression, it experiences an altered social and endocrine milieu. However, it is not yet known whether these endocrine changes involve the GnRH-1 and/or kisspeptin system. In this present study, GnRH-1- and kisspeptin-immunoreactivity were analysed in eight groups of naked mole-rats; breeders, reproductively-activated non-breeders, GDX breeders and subordinates of both sexes to elucidate their potential roles in HPG axis activation. The effects of reproductive status, breeding history, gonadectomy and sex were also assessed. Furthermore, plasma testosterone, oestradiol and progesterone levels of all eight animal groups were analysed to determine any significant differences between the groups. Adrenal oestradiol levels were also measured to determine the physiological source of circulating oestradiol.

MATERIALS AND METHODS

Study animals

The current experiment compared eight groups of naked mole-rats: (group 1) female breeders (N= 6); (group 2) male breeders (N= 6); (group 3) female reproductively-activated non-breeders (N= 7); (group 4) male reproductively-activated non-breeders (N= 6); (group 5) female GDX breeders (N= 5); (group 6) male GDX breeders (N= 5); (group 7) female subordinates (N= 11) and (group 8) male subordinates (N= 14). Not all the individual animals were used for all the experiments due to tissue damage. The precise numbers of animals studied in individual experiments are mentioned in the results section.

Steroidal hormone assays

Adrenals were taken out, cleaned and weighed. Adrenal pairs of each animal were incubated in 2 ml of Krebs–Ringer's buffer (pH 7.4) at 37°C for 1.5 hr in an atmosphere of 95% O2 and 5% CO_2 in a shaking water bath. Adrenal tissue was homogenised in a tight glass homogeniser in 0.01 M phosphate buffer (pH 7.4), the homogenate was centrifuged for 10 minutes, the lipid layer was discarded and supernatant (tissue extract) was taken by pipettes. The medium was then collected and stored at −20°C for oestradiol assays.

Plasma testosterone, oestradiol (plasma and adrenal) and progesterone were analysed by radioimmunoassay following ether extraction and chromatographic isolation (Wingfield and Farner, 1975; Lovern and Wade, 2003). For each sample, 32-50 µl of plasma or adrenal medium (recorded to the nearest µl for each sample) in 0.5 ml ddH_2O was equilibrated overnight at 4°C with 1000 cpm of ^{3}H-testosterone (NET-370, 70 Ci/mmol) or ^{3}H-oestradiol (NET-317, 72 Ci/mmol) for the testosterone and oestradiol assays respectively, and ^{3}H-progesterone (NET-381, 102 Ci/mmol) for the progesterone assay; all radioisotopes were from PerkinElmer, Inc. (Boston, MA). Samples were then extracted twice with 2 ml diethyl ether, dried in a 37°C water bath with the aid of nitrogen gas, and then reconstituted in 0.5 ml of 10% ethyl acetate in isooctane (for testosterone and oestradiol assays) or ethylene glycol pseudosaturated in isooctane (progesterone assay) for column chromatography. Columns were packed with a diatomaceous earth:ethylene glycol:propylene glycol upper phase (4:1:1 m:v:v) and a diatomaceous earth:ddH_2O (3:1 m:v) lower phase. Diatomaceous earth (“Celite”) was from Sigma-Aldrich (St. Louis, MO). In the testosterone and oestadiol assays, neutral lipids were removed with 1.5 ml isooctane, and testosterone and oestradiol with 2 ml 20% and 2.5 ml 40% ethyl acetate in isooctane, respectively. In the progesterone assay, the neutral lipid fraction was eluted with 1.25 ml isooctane and discarded, and

progesterone was eluted with 2 ml 5% ethyl acetate in isooctane. All samples were dried in a 37°C water bath with nitrogen, resuspended in phosphate buffer, and stored at 4°C overnight.

Competitive binding radioimmunoassays were performed using the appropriate tritiated steroid tracer (see above), antibodies from Wien Laboratories for testosterone (T-3003; Succasunna, NJ), Biogenesis for oestradiol (7010-2650; Kingston, NH) and Sigma-Aldrich for progesterone (P5289), and steroid standards from Sigma-Aldrich. Standard curves ranged from 0.98 to 250 pg and were run in triplicate. Samples were run singly and adjusted for individual recovery and initial sample volume. Average recovery for testosterone, oestradiol and progesterone was 68%, 71% and 69%, respectively. The intra-assay coefficient of variation, based on four aliquots from a standard pool for each steroid, was 9% for testosterone, 7% for oestradiol and 18% for progesterone.

GnRH-1 and kisspeptin-immunohistochemistry

One of the series of sections was immunostained for GnRH-1 and another one was immunostained for kisspeptin. After washing in 0.1 M PBS (pH7.4), free-floating sections were treated with 0.1% sodium borohydride (Sigma-Aldrich, Dorset, UK) for 30 min to neutralise the acrolein immersion fixation, washed in 0.1 M PBS (pH7.4) and treated with 0.5% Triton X-100 to increase cell permeability. Endogenous peroxidase was suppressed using 1% hydrogen peroxide (30 min). The sections were briefly rinsed in 0.1 M PBS and incubated in 2% normal donkey serum overnight at 4^{o}C. The sections were then incubated in either monoclonal mouse anti-GnRH-1 (1:15,000; Millipore, MA, USA; N-terminus clone HU11B, raised against mouse GnRH-1, QHWSYGLRPG, conjugated to bovine thyroglobulin) or polyclonal rabbit anti-kisspeptin (1:30,000; A. Caraty no. 564, raised against mouse kisspeptin-10 YNWNSFGLRY-NH2; (Franceschini *et al.*, 2006) for 48 hours at 4^{o}C. After a PBS wash, the sections were incubated in either biotinylated donkey anti-mouse IgG (for mouse antibody) (each at 1:1000; Stratech, Newmarket, Suffolk, UK) or biotinylated donkey anti-rabbit IgG (for rabbit antibody) for two hours at room temperature. Following a wash in PBS, the tissue was incubated in an avidin-biotin peroxidase complex (1:1000, Elite Kit; Vector Laboratories, Burlingame, CA, USA) for 90 minutes at room temperature. After the sections had been transferred to 0.05 M TRIS buffer, immunoreactivity was visualised with diaminobenzidine and 0.15% ammonium nickel sulphate, and activated by 0.05% H_2O_2 to visualise GnRH-1- or kisspeptin-immunoreactivity. The sections were mounted from elvanol (polyvinyl alcohol) solution onto glass slides. After cover-slips had been applied over DPX mountant (Sigma-

Aldrich, Dorset, UK), and the slides were coded so that the investigator was blind to the status of each subject.

Controls and specificity

Control procedure included omission of the primary GnRH-1 or kisspeptin antibodies, pre-adsorbing the GnRH-1 antibody overnight with GnRH-1 peptide 1 µg/ml (pEHWSYGLRPG-NH2; Phoenix Pharmaceuticals) or pre-adsorbing the kisspeptin antibodies overnight with kisspeptin-10 mouse peptide 1 µg/ml (YNWNSFGLRY-NH2; Phoenix Pharmaceuticals, Peterborough, UK) to determine whether the kisspeptin antibody cross-reacts with other RFamide peptides, it was incubated with RFRP-1 peptide 10 µg/ml (VPHSAANLPLRF-NH2, Phoenix Pharmaceuticals) or RFRP-3 peptide 10 µg/ml (VPNLPQRF- NH2, Phoenix Pharmaceuticals). Both the GnRH-1 and kisspeptin antibodies have been previously characterised to determine sequence specificity to GnRH-1 (Urbanski, 1991) and kisspeptin (Franceschini *et al.*, 2006) peptide molecules, respectively. The GnRH-1 antibody was able to bind to various radioiodinated GnRH-1 fragments (even one as short as four amino acid residues in length) yet failed to show significant binding to the deamidated form of the decapeptide (GnRH-1 free acid), the precursor molecule (pro-GnRH-1) nor to other hypothalamic peptides (such as, growth hormone-releasing hormone, oxytocin, somatostatin, thyrotrophin-releasing hormone and vasoactive intestinal peptide) (Urbanski, 1991). It therefore appears to show high sequential immunospecificity to the GnRH-1 molecule. The kisspeptin antibody was able to bind to radioiodinated mouse kisspeptin-10, yet failed to show significant binding to other hypothalamic peptides (such as, GnRH, galanin, substance P, neuropeptide Y, corticotrophin releasing hormone, alphamelanocyte stimulating hormone, somatostatin and prolactin releasing peptide), It therefore appears to show high sequence immunospecificity to the kisspeptin-10 molecule YNWNSFGLRY-NH2 (Franceschini *et al.*, 2006).

The distribution of its immunoreactivity in the present study does not differ qualitatively from that observed for GnRH-1 in other mammals (see Discussion). This is consistent with our discovery that the mole-rat GnRH-1 amino acid sequence deduced from its precursor transcript is identical to the decapeptide that has been identified in all mammals apart from guinea pigs (Kalamatianos *et al.*, 2005). Although mole-rats possess a GnRH-1 gene that contains some of the nucleotide substitutions found in guinea pigs, with which mole-rats share remote ancestry, these differences do not result in the amino acid substitutions (at positions 2 and 7) that distinguish guinea pig GnRH-1 from the common form (Kalamatianos *et al.*, 2005).

Digital photomicrographs

Brightfield photomicrographs were obtained using a Nikon E600 microscope at magnifications of x40, x100 and x200 with a Micro-Publisher 5.0 camera (InterFocus Imaging, Cambridge, UK). Photomicrographs of thionin stained sections were taken using a precision illuminator (Northern Light model R95; Interfocus Imaging) with a CoolSnap CF camera (Photometrics, Marlow, UK). Both cameras were controlled by MCID Core software (Interfocus Imaging). Images were later post-processed using Adobe Photoshop, CS3 to adjust brightness and contrast. Background artefacts were removed as necessary; no other modifications were made to images. Final images were compiled into multi-panel plates in Microsoft Publisher, minor changes to brightness and contrast were made after importing into Microsoft Publisher if necessary.

Quantification and statistical analysis

Slides were examined and counted under brightfield illumination using a Nikon E600 microscope at X200 magnification. For each animal, the number of GnRH-1-ir cell bodies was counted throughout the septo-preoptico-hypothalamo pathway and multiplied by 4 to obtain a total number (since brain sections were originally cut into a series of 4). The number of kisspeptin-ir cell bodies was counted throughout the RP3V and PVH, and multiplied by 4 to obtain a total number for each area. For each animal group, the mean number of kisspeptin-ir cell bodies in the caudal Arc (cArc) and DMH was established using anatomically matched brain sections. For the Arc, the analysis focused on the caudal part of the nucleus because the dense plexus of kisspeptin-ir processes more rostrally obscured the kisspeptin-ir cell bodies. The final number of animals per group for each measure is presented in the figures.

The density of GnRH-1-immunoreactivity in the ME for female breeders (group 1, n= 4), female subordinates (group 7, n= 4), male breeders (group 2, n= 4) and male subordinates (group 8, n= 4) were quantified as the mean area occupied by GnRH-1-ir processes according to the method previously described (Molteno *et al.*, 2004). Statistical differences between the four groups were assessed by an overall one-way (IBM SPSS, Version 20). Data are presented as mean ± standard error of the mean (SEM). The density of immunoreactivity in the ME of the other four animal groups were not analysed due to lack of available tissue.

For all comparisons, statistical significance was set at $p < 0.05$. Some animals were excluded from certain comparisons because of tissue damage in specific areas. For each dependent measure, an overall one-way ANOVA was performed including all eight animal groups. If the overall one-way ANOVA test was statistically significant,

then each animal group was compared with one-another using a post-hoc Tukey test. A one-way ANOVA test was performed on:

- The body masses (g)
- The total number of GnRH-1-ir cell bodies
- The percentage of GnRH-1-ir cell bodies rostral and caudal to the SCN
- The number of kisspeptin-ir cell bodies in the RP3V
- The number of kisspeptin-ir cell bodies in the PVH
- The mean number of kisspeptin-ir cell bodies in the cArc
- The mean number of kisspeptin-ir cell bodies in the DMH
- The plasma testosterone level (ng/ml)
- The plasma oestradiol level (ng/ml)
- The plasma progesterone level (ng/ml)
- The adrenal oestradiol level (pg/mg)
- The mass of the adrenal glands (mg)

A series of independent samples T-tests was performed to elucidate whether: (**1**) breeding/reproductively-activated animals (groups 1-6) are significantly different to subordinate animals (groups 7-8), (**2**) breeders with litter (groups 1-2) are significantly different to reproductively-activated animals with no litter (groups 3-4), (**3**) gonadally-intact breeders (groups 1-2) are significantly different to GDX breeders (groups 5-6), and (**4**) females (groups 1, 3, 5 and 7) are significantly different to males (groups 2, 4, 6 and 8). An independent samples T-test testing the four specific hypotheses were performed on:

- The body mass (g)
- The total number of GnRH-1-ir cell bodies
- The number of kisspeptin-ir cell bodies in the RP3V
- The number of kisspeptin-ir cell bodies in the PVH
- The mean number of kisspeptin-ir cell bodies in the cArc
- The mean number of kisspeptin-ir cell bodies in the DMH

A Pearson's correlation was done to determine whether there were statistically significant correlations between:

- Number of GnRH-1-ir cell bodies and body mass (g)
- Number of RP3V kisspeptin-ir cell bodies and body mass (g)
- Number of PVH kisspeptin-ir cell bodies and body mass (g)
- Number of cArc kisspeptin-ir cell bodies and body mass (g)
- Number of DMH kisspeptin-ir cell bodies and body mass (g)

- Number of RP3V kisspeptin-ir cell bodies and the total number of GnRH-1-ir cell bodies
- Number of PVH kisspeptin-ir cell bodies and the number of GnRH-1-ir cell bodies
- Number of cArc kisspeptin-ir cell bodies and the total number of GnRH-1-ir cell bodies
- Number of DMH kisspeptin-ir cell bodies and the total number of GnRH-1-ir cell bodies
- Number of RP3V kisspeptin-ir cell bodies and plasma testosterone level (ng/ml)
- Number of RP3V kisspeptin-ir cell bodies and plasma oestradiol level (ng/ml)
- Number of RP3V kisspeptin-ir cell bodies and plasma progesterone level (ng/ml)
- Number of PVH kisspeptin-ir cell bodies and plasma testosterone level (ng/ml)
- Number of PVH kisspeptin-ir cell bodies and plasma oestradiol level (ng/ml)
- Number of PVH kisspeptin-ir cell bodies and plasma progesterone level (ng/ml)
- Number of cArc kisspeptin-ir cell bodies and plasma testosterone level (ng/ml)
- Number of cArc kisspeptin-ir cell bodies and plasma oestradiol level (ng/ml)
- Number of cArc kisspeptin-ir cell bodies and plasma progesterone level (ng/ml)
- Number of DMH kisspeptin-ir cell bodies and plasma testosterone level (ng/ml)
- Number of DMH kisspeptin-ir cell bodies and plasma oestradiol level (ng/ml)
- Number of DMH kisspeptin-ir cell bodies and plasma progesterone level (ng/ml)
- Plasma oestradiol level (ng/ml) and adrenal oestradiol level (pg/mg)
- Adrenal mass (mg) and body mass (g)
- Adrenal oestradiol level (pg/mg) and adrenal mass (mg)

RESULTS

Tests for specificity of the immunoreactivity

Pre-adsorption of the GnRH-1 antibody with the GnRH-1 peptide removed all immunoreactivity from the naked mole-rat brain (Plate 3.1A). Similarly, pre-adsorption of the kisspeptin antibody (A. Caraty no. 564) with the mouse kisspeptin-10 peptide resulted in a complete absence of immunoreactivity in the naked mole-rat brain (Plate 3.1B). In contrast, pre-adsorption of the kisspeptin antibody with either the RFRP-1 or the RFRP-3 peptide resulted in no attenuation of the signal (Plate 3.1C-D). Kisspeptin-immunoreactivity in the DMH was neutralised by the kisspeptin-10 peptide, but not by the RFRP-1 or RFRP-3 peptide. These results indicate that the kisspeptin and GnRH-1 antibodies used in this study were only specifically staining for kisspeptin and GnRH-1 neurones, respectively.

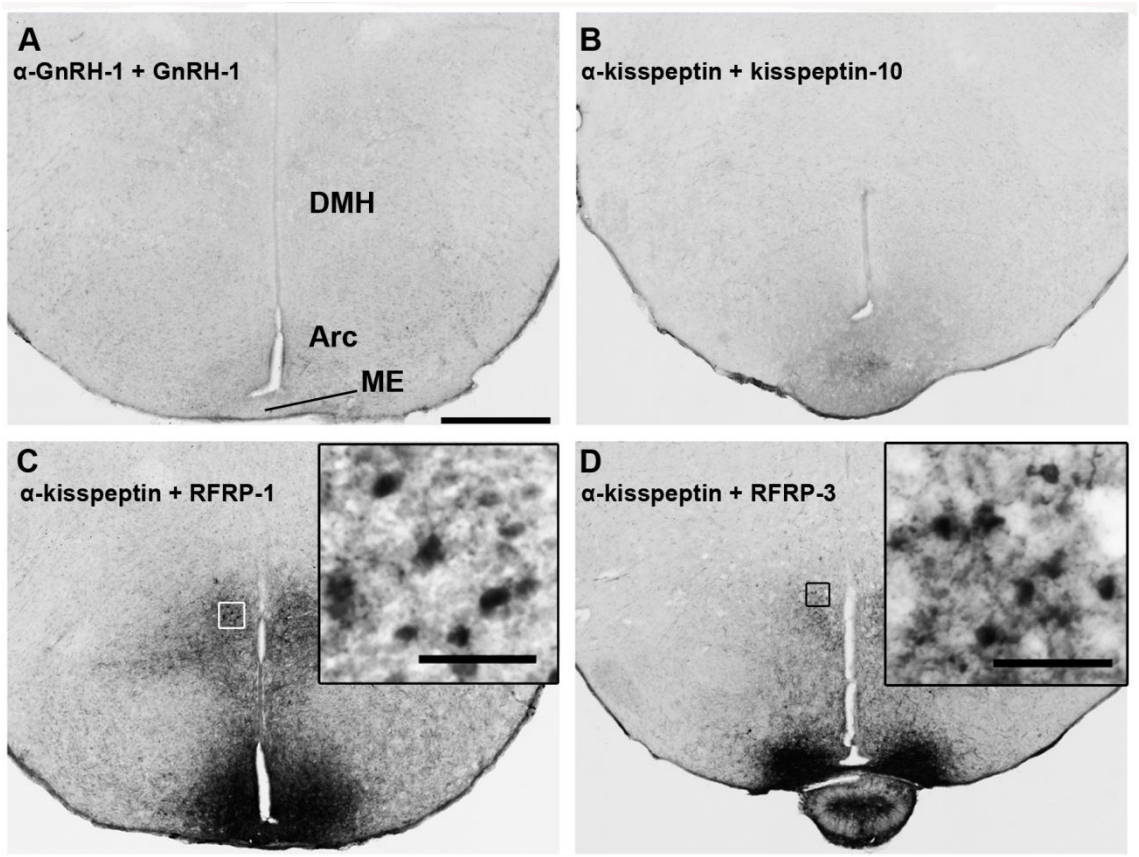


Plate 3.1: Brightfield photomicrographs of representative coronal sections showing staining with (A) the GnRH-1 antibody (α-GnRH) pre-adsorbed with GnRH-1 peptide, (B) the kisspeptin antibody (α-kisspeptin) pre-adsorbed with kisspeptin-10 peptide, (C) the kisspeptin antibody pre-adsorbed with RFRP-1 peptide or (D) the kisspeptin antibody pre-adsorbed with RFRP-3 peptide. Boxed regions are shown at higher magnification by insets within the same photomicrograph. Arc, arcuate nucleus; DMH, dorsomedial hypothalamic nucleus; ME, median eminence. Scale bars = 500 µm in A (applies to A-D); 50 µm in the insets for C & D.

Body masses

Breeding/reproductively-activated animals (groups 1-6) had a mean mass of 40.19 g ± 1.93 (SEM), subordinate animals (groups 7-8) had a mean mass of 33.21 g ± 1.78 (SEM). The female GDX breeders (group 5) were the heaviest (Table 3.1). One-way ANOVA revealed a significant effect of animal group on body mass; $F(7,47)= 4.155$, $p= 0.001$. Planned contrasts indicated that female GDX breeders (group 5) were significantly heavier than male breeders (group 2, $p= 0.015$), activated non-breeding males (group 4, $p= 0.028$), females subordinates (group 7, $p= 0.023$) and male subordinates (group 8, $p= 0.006$).

Breeding/reproductively-activated animals (groups 1-6) were significantly heavier than subordinate animals (groups 7-8); $t(53)= 2.416$, $p= 0.019$. Breeders (groups 1-2) were not significantly different in mass compared to reproductively-activated animals (groups 3-4); $t(23)= 0.550$, $p= 0.588$. GDX animals (groups 5-6) were significant heavier than intact breeders (groups 1-2; $t(20)= 2.746$, $p= 0.012$. Females (groups 1, 3, 5 and 7) were not significantly different in mass compared to males (groups 2, 4, 6 and 8); $t(53)= 1.843$, $p= 0.071$.

Table 3.1: Mean body mass of naked mole-rats (g) (± SEM)

	Animal Group	Sex	N	Mean body mass (g)
1	Breeder	F	6	43.43 ± 4.29
2	Breeder	M	6	31.75 ± 1.48 ^{B}
3	Activated non-breeder	F	6	37.55 ± 4.21
4	Activated non-breeder	M	7	33.64 ± 3.70 ^{B}
5	GDX breeder	F	5	51.66 ± 5.77 ^{A}
6	GDX breeder	M	5	47.28 ± 3.88
7	Subordinate	F	10	34.43 ± 3.17 ^{B}
8	Subordinate	M	10	31.98 ± 1.72 ^{B}

For the mean body mass, statistically significant differences ($p< 0.05$) exist where superscript letters differ.

Distribution of GnRH-1-cell bodies

GnRH-1-ir cell bodies were mostly distributed in a loose continuum along the septo-preoptico-hypothalamo pathway from the MS to the OVLT, with a few caudal to the SCN (Plate 3.2A1-E2). Dorsally, the GnRH-1-ir cells were located close to the midline with their long axis in the dorsoventral orientation (Plate 3.3B-C). Ventrally, the GnRH-1-ir cells were located surrounding the third ventricle (3V) and were distributed laterally with their long axis in the mediolateral orientation (Plate 3.3D). The vast majority of the GnRH-1-ir cell bodies were located in the MS, VDB, HDB, MPOA or OVLT, rostral to

the SCN (mean= 95%, SEM= 0.62). Isolated GnRH-1-ir cell bodies were present in the retrochiasmatic region (RCh, Plate 3.3G), lateral to the RCh (Plate 3.2G2), in the medial basal hypothalamus (MBH, Plate 3.2K2 inset) and dorsal to the hypothalamus (Plate 3.2K2), caudal to the SCN (mean= 5%, SEM= 0.62). There were no significant differences in the percentage of GnRH-1-ir cell bodies rostral to the SCN between the 8 animal groups; $F(7,30)= 1.93$, $p= 0.099$ (Table 3.2,). There were no significant differences in the percentage of GnRH-1-ir cell bodies caudal to the SCN between the 8 animal groups; $F(7,30)= 1.93$, $p= 0.099$ (Table 3.2).

Distribution of GnRH-1-ir processes

GnRH-1-ir processes were present loosely along the septo-preoptico-hypothalamo continuum from the MS and the VDB+HDB, to the OVLT and MPOA, and finally to the ME (Plate 3.2A1-K2). GnRH-1-ir processes were also scattered in the ventrolateral septum (VLS) close to the lateral ventricle (LV, Plate 3.3A). Dorsally, many of these GnRH-1-ir processes coursed in a dorsoventral orientation close to the midline (Plate 3.2A1-D2). Ventrally, the GnRH-1-ir processes were distributed laterally along the base of the hypothalamus (Plate 3.3C). Scattered GnRH-1-ir processes were observed throughout the MPOA and median preoptic nucleus (MPO; Plate 3.2C1). A dense network of GnRH-1-ir processes was seen around and within the OVLT and lateral to the 3V (Plates 3.2C1-C2 & 3.3C-D). GnRH-1-ir processes were also present in the midline within the vestigial optic chiasm (OCh), which forms the floor of the 3V (Plate 3.3E & inset). Diffuse GnRH-1-ir processes were located dorsal, lateral and caudal to the SCN, but well-nigh absent from this nucleus (Plates 3.2F1-G2 & 3.3F-G). More caudally, they were largely restricted to the Arc region and followed a rostrocaudal course into the ME where they form an extensive arching pattern from the borders of the 3V, achieving a dense aggregation in the ME across its breadth (Plates 3.2H1-I2 & 3.4). The dense GnRH-1-immunoreactivity in the external zone of the ME continues caudally into the head of the pituitary stalk (PS) forming a dense congregation (Plate 3.2J1-J2). There were no differences in the distribution of GnRH-1-ir cell processes between any of the eight animal groups.

No differences were observed in the distribution of GnRH-1-ir processes between all eight animal groups. Analysis of the density of GnRH-1-immunoreactivity within the ME failed to identify significant differences ($F3,15= 0.588$, $p= 0.634$) between female breeders (15740.75 ± 3283.77 μm^2, Plate 3.4A), female subordinates (19515.00 ± 1603.24 μm^2, Plate. 3.4B), male breeders (16162.75 ± 2259.98 μm^2, Plate 3.4C) and male subordinates (18383.25 ± 1889.56 μm^2, Plate 3.4D).

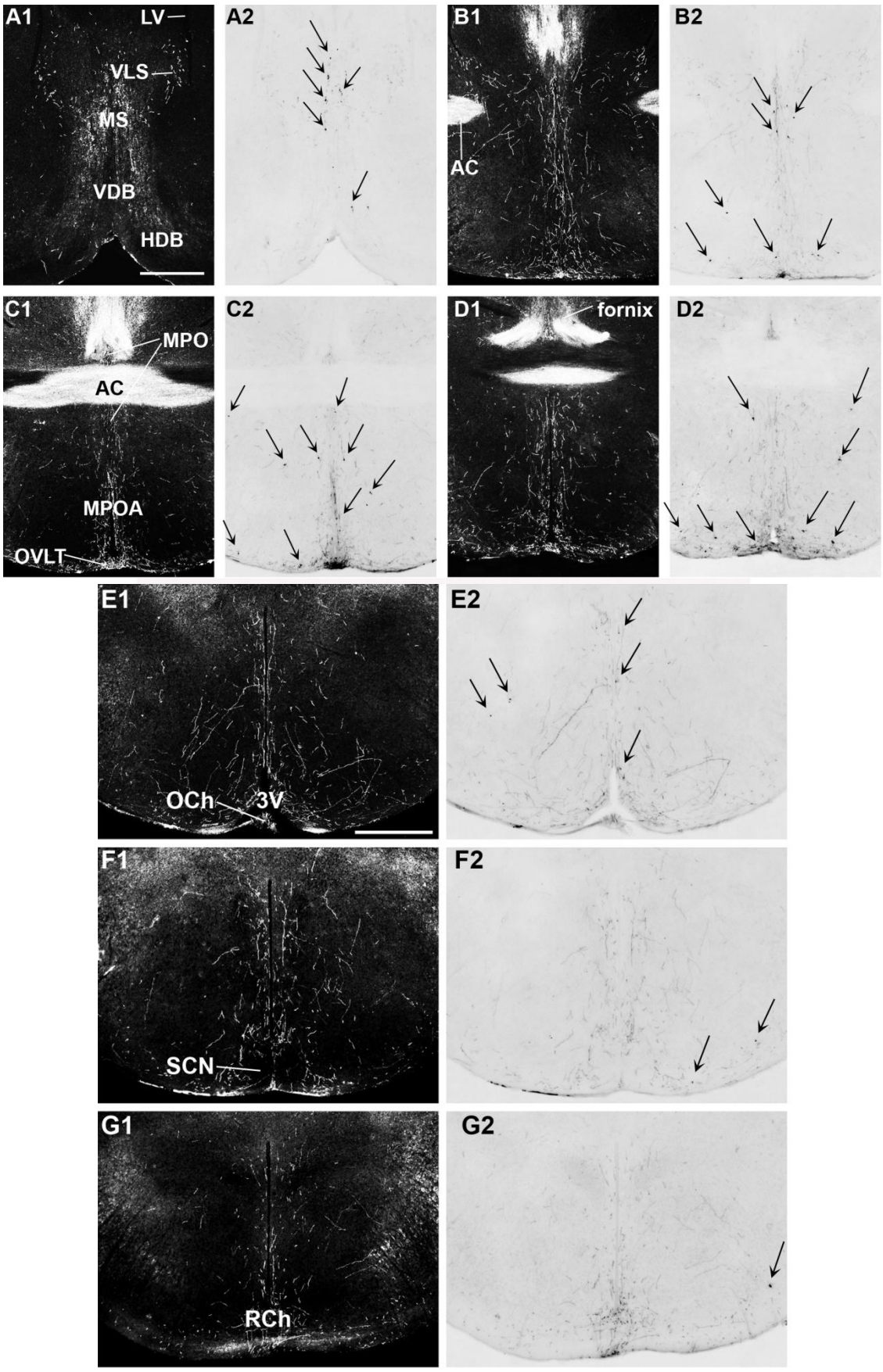


Plate 3.2
Continued overleaf…

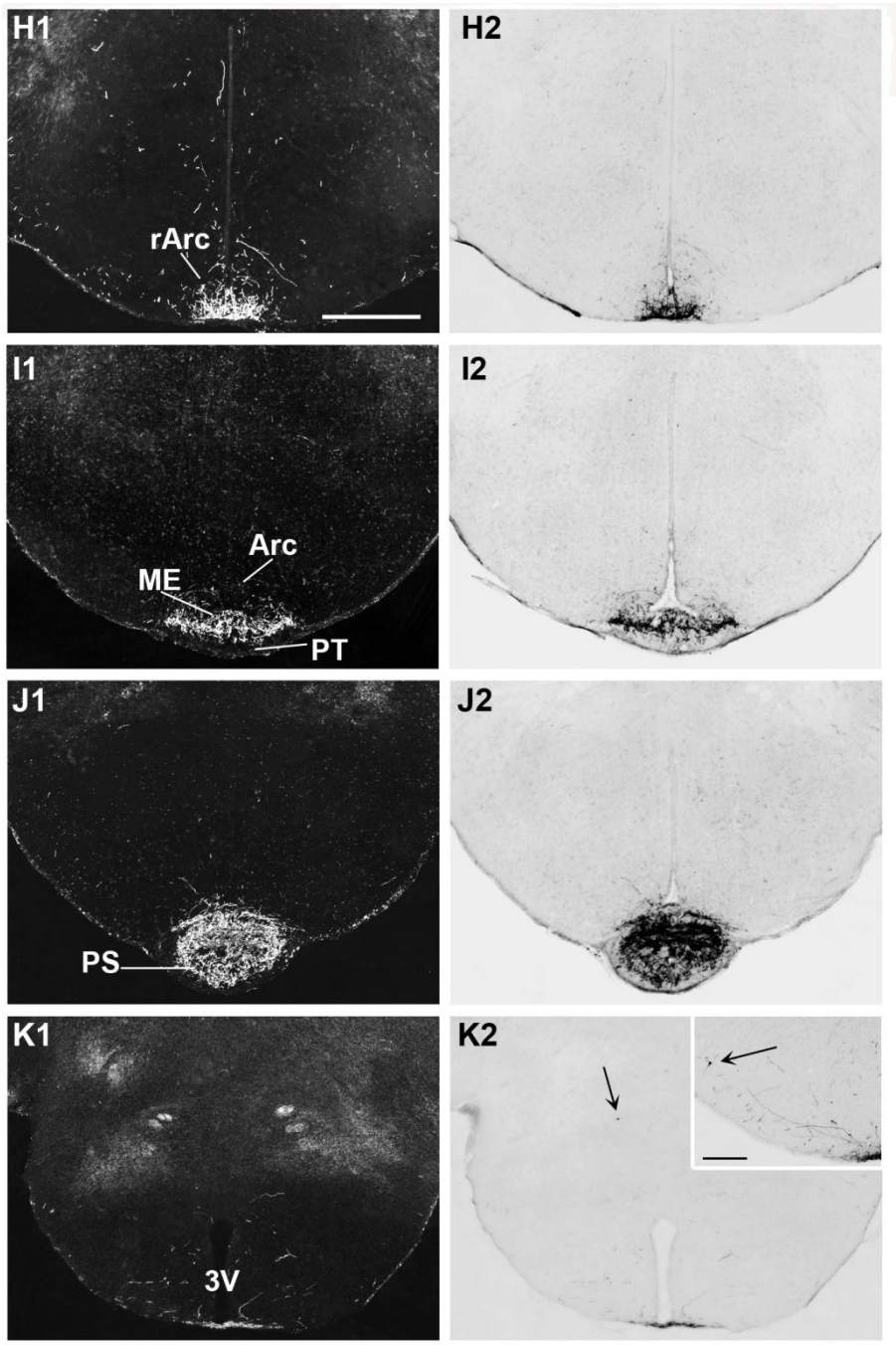


Plate 3.2: Low magnification (x40) darkfield and brightfield photomicrographs of representative coronal sections (A1-K2; rostral to caudal) showing GnRH-1 immunoreactivity in the brain of a reproductive female naked mole-rat. 3V, third ventricle; AC, anterior commissure; Arc, arcuate nucleus; HDB, horizontal limb of the diagonal band; LV, lateral ventricle, ME, median eminence; MPO, median preoptic nucleus, MPOA, medial preoptic area, MS, medial septum; OCh, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; PS, pituitary stalk; PT, pars tuberalis; rArc, rostral arcuate nucleus; RCh, retrochiasmatic area; SCN, suprachiasmatic nucleus; VDB, vertical limb of the diagonal band; VLS, ventrolateral septum. Boxed inset in K2 shows the medial basal hypothalamus (MBH). Arrows indicate selected GnRH-1-ir cell bodies. Scale bars = 500 µm in A1, E1 & H1 (applies to all images); 200 µm in the inset for K2.

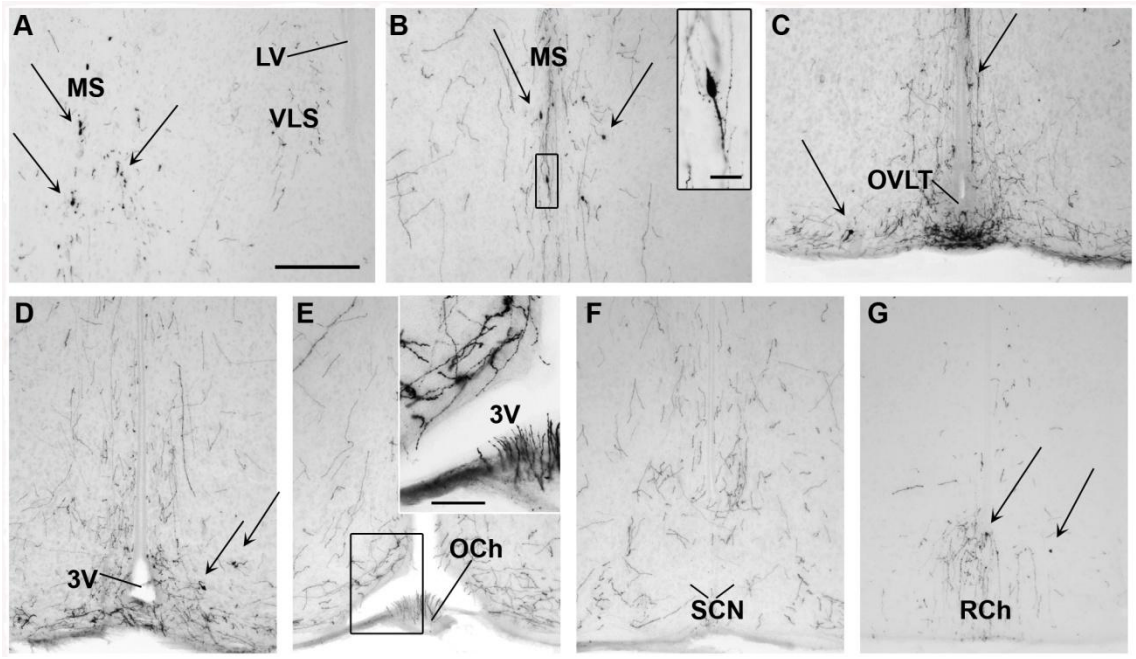


Plate 3.3: High magnification (x100) brightfield photomicrographs of representative coronal sections (A-G; rostral to caudal) showing GnRH-1-immunoreactivity in the brain of a female reproductive naked mole-rat. Boxed regions are shown at higher magnification by insets within the same photomicrograph. 3V, third ventricle; LV, lateral ventricle; MS, medial septum; OCh, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; RCh, retrochiasmatic area; SCN, suprachiasmatic nucleus; VLS, ventrolateral septum. Arrows indicate selected GnRH-1-ir cell bodies. Scale bars = 200 µm in A (applies to A-G); 20 µm in the inset for B; 40 µm in the inset for E.

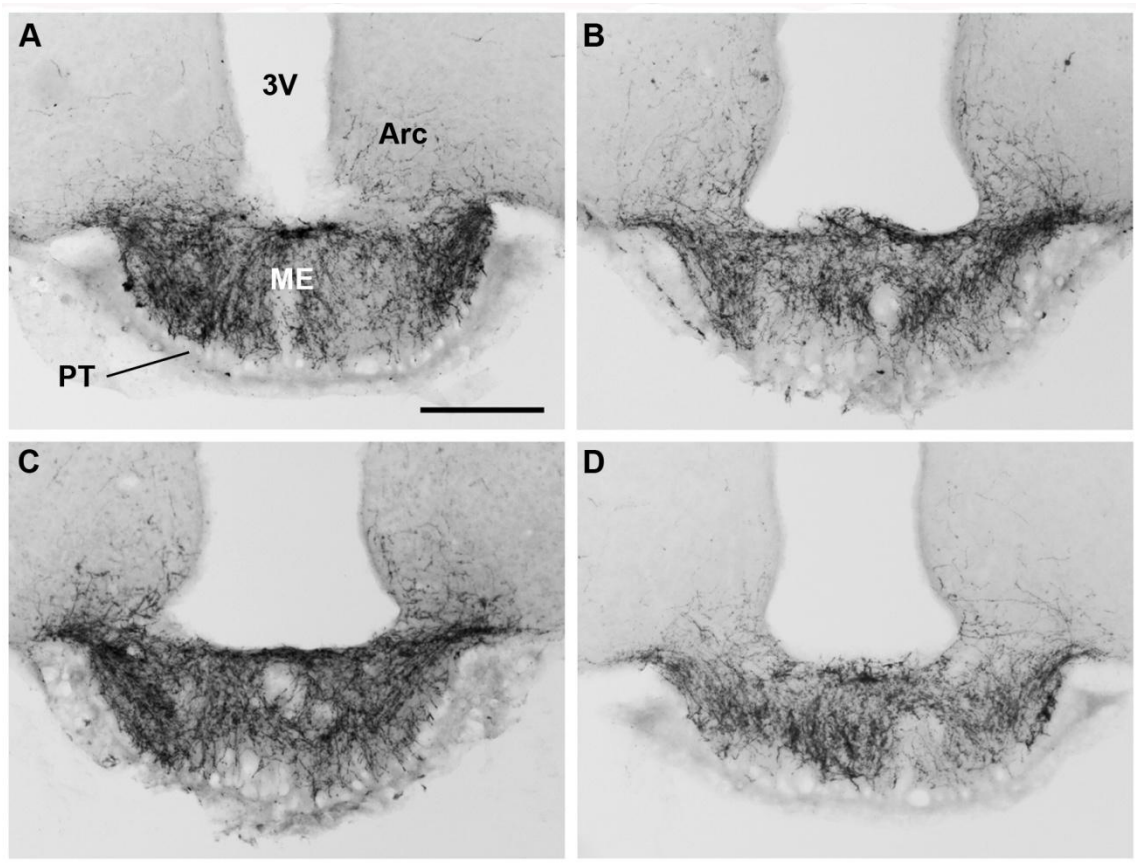


Plate 3.4: High magnification (x100) brightfield photomicrographs of representative median eminences showing GnRH-1-immunoreactivity in a (A) breeding female (group 1), (B) subordinate female (group 7), (C) breeding male (group 2) and (D) subordinate male (group 8) naked mole-rat. 3V, third ventricle; Arc, arcuate nucleus; PT, pars tuberalis; ME, median eminence. Scale bar = 200 µm in A (applies to A-D).

Total number of GnRH-1-ir cell bodies

There was a large variation in the total number of GnRH-1-ir cells in the brains of all eight animal groups studied (Figure 3.1). The total numbers of GnRH-1-ir cell bodies in breeding/reproductively-activated animals (groups 1-6) ranged from 76 to 588 whilst the numbers of GnRH-1-ir cell bodies in subordinate animals (groups 7-8) ranged from 84 to 294. One-way ANOVA revealed a significant effect of animal group on the total number of GnRH-ir cell bodies; $F(7,40)= 2.362$, $p= 0.041$. Planned contrasts indicated that reproductively-activated females (group 3) had significantly greater total number of GnRH-1-ir cell bodies than GDX breeding males (group 6, $p= 0.046$).

Overall, there were no significant differences in the total number of GnRH-1-ir cells between the breeding/reproductively-activated animals (groups 1-6) and the subordinate animals (groups 7-8); $t(43.39)= 1.023$, $p= 0.251$. There were no significant differences in the number of GnRH-1-ir cells between breeders (groups 1-2) and reproductively-activated non-breeders (groups 3-4); $t(50)= 1.022$, $p= 0.312$). There were no significant differences between breeders (groups 1-2) and GDX breeders (groups 5-6); $t(15)= 0.182$, $p= 0.858$. Furthermore, there were no significant differences in the number of GnRH-1-ir cell bodies between females (groups 1, 3, 5 and 7) and males (groups 2, 4, 6 and 8); $t(50)= 1.403$, $p= 0.167$. There was no significant correlation between body mass and number of GnRH-1-ir cell bodies ($r= 0.036$, $p= 0.820$, Figure 3.2).

Table 3.2: The percentage (± SEM) of GnRH-1-ir cell bodies rostral and caudal to the SCN.

	Animal Group	Sex	N	% GnRH-1 cell bodies rostral to SCN	% GnRH-1 cell bodies caudal to SCN
1	Breeder	F	6	94.85 ± 1.58 ^{A}	5.15 ± 1.58 ^{B}
2	Breeder	M	5	98.74 ± 0.78 ^{A}	1.26 ± 0.78 ^{B}
3	Activated non-breeder	F	6	93.68 ± 1.24 ^{A}	6.32 ± 1.24 ^{B}
4	Activated non-breeder	M	6	93.17 ± 1.44 ^{A}	6.83 ± 1.44 ^{B}
5	GDX breeder	F	3	91.53 ± 2.79 ^{A}	8.47 ± 2.79 ^{B}
6	GDX breeder	M	3	96.37 ± 1.86 ^{A}	3.63 ± 1.86 ^{B}
7	Subordinate	F	5	94.48 ± 2.03 ^{A}	5.52 ± 2.03 ^{B}
8	Subordinate	M	4	97.45 ± 1.47 ^{A}	2.55 ± 1.47 ^{B}

For the percentage of GnRH-1 cell bodies rostral and caudal to the SCN, statistically significant differences ($p< 0.05$) exist where superscript letters differ. SCN, suprachiasmatic nucleus

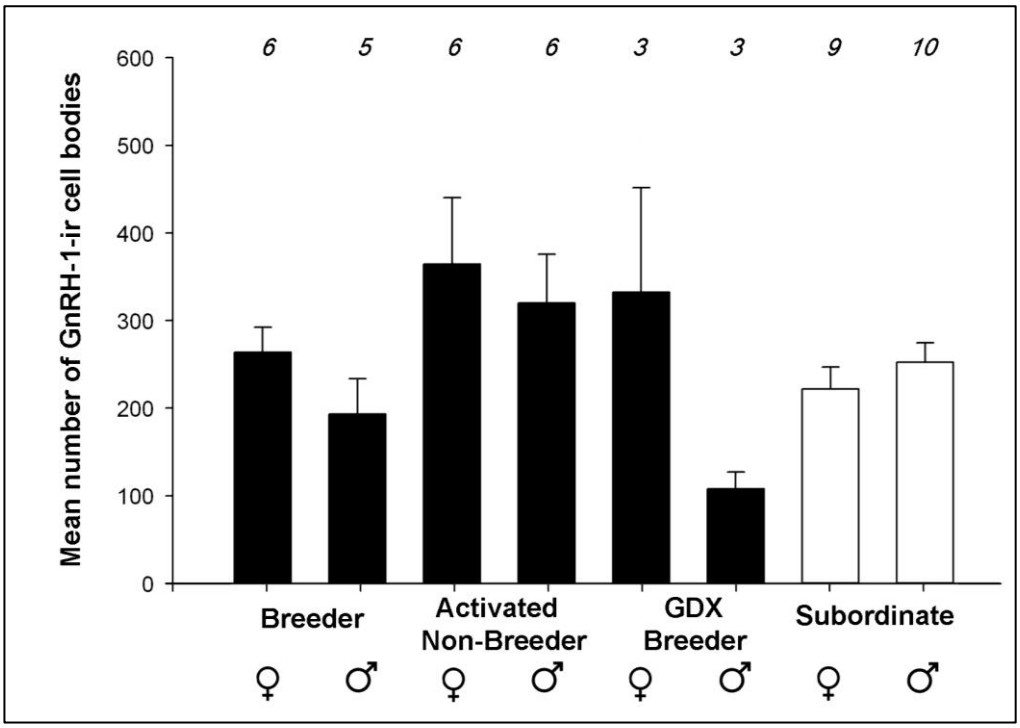


Figure 3.1: Mean (± SEM) number of GnRH-1-ir cell bodies counted throughout the brains of breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and male) and subordinates (female and male). Number of animals per group is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; white bars represent subordinate groups.

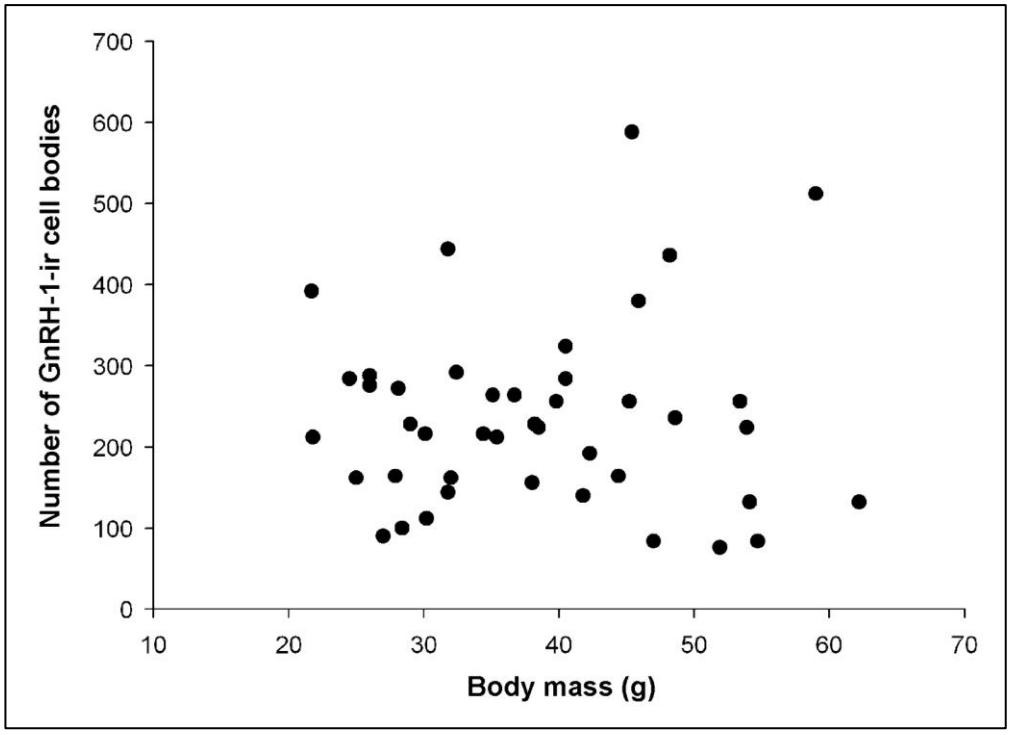


Figure 3.2: Lack of significant correlation between body mass (g) and total number of GnRH-1-ir cell bodies.

Distribution of kisspeptin-ir cell bodies

Four distinct populations of kisspeptin-ir cell bodies were found in the naked mole-rat brain. The two largest populations were the RP3V (consisting of the AVPV and PeN, Plates 3.5C-F & 3.6 C-E) and PVH (Plate 3.5G-I & 3.6F-G). Within the RP3V, kisspeptin-ir cell bodies were spread throughout the entirety of the AVPV, with some cells extending laterally and dorsally (Plate 3.6C-D), but in the PeN, they were located close to the wall of the 3V forming a tight band of cells (Plate 3.6E). In the PVH, kisspeptin-ir cell bodies were distributed across the breadth of the PVH (Plate 3.6F-G). There was a distinct absence of kisspeptin-ir cell bodies in the SCN (Plates 3.5G-H & 3.6F-G), though kisspeptin-ir cell bodies were scattered in the RCh just caudal to the SCN (Plates 3.5I-J & 3.6I). The next most numerous population was located in the Arc, surrounded by a dense network of kisspeptin-ir processes (Plates 3.5K-N & 3.6K-L). Kisspeptin-ir cell bodies in the Arc region were largely contained within the Arc and did not extend ventrally down into the ME (Plate 3.6K). Kisspeptin-ir cell bodies were also found at low density throughout the DMH (Plates 3.5K-L & 3.6J). There was a distinct absence of kisspeptin-ir cell bodies in the VMH, even though kisspeptin-ir cell bodies were abundant in the Arc (Plates 3.5K-L). There were a few scattered kisspeptin-ir cell bodies in the medial mammillary nucleus (MM, Plates 3.5O & 3.6M). Kisspeptin-ir cell bodies were not detected elsewhere. There were no differences in the distribution of kisspeptin-ir cell bodies between any of the eight animal groups.

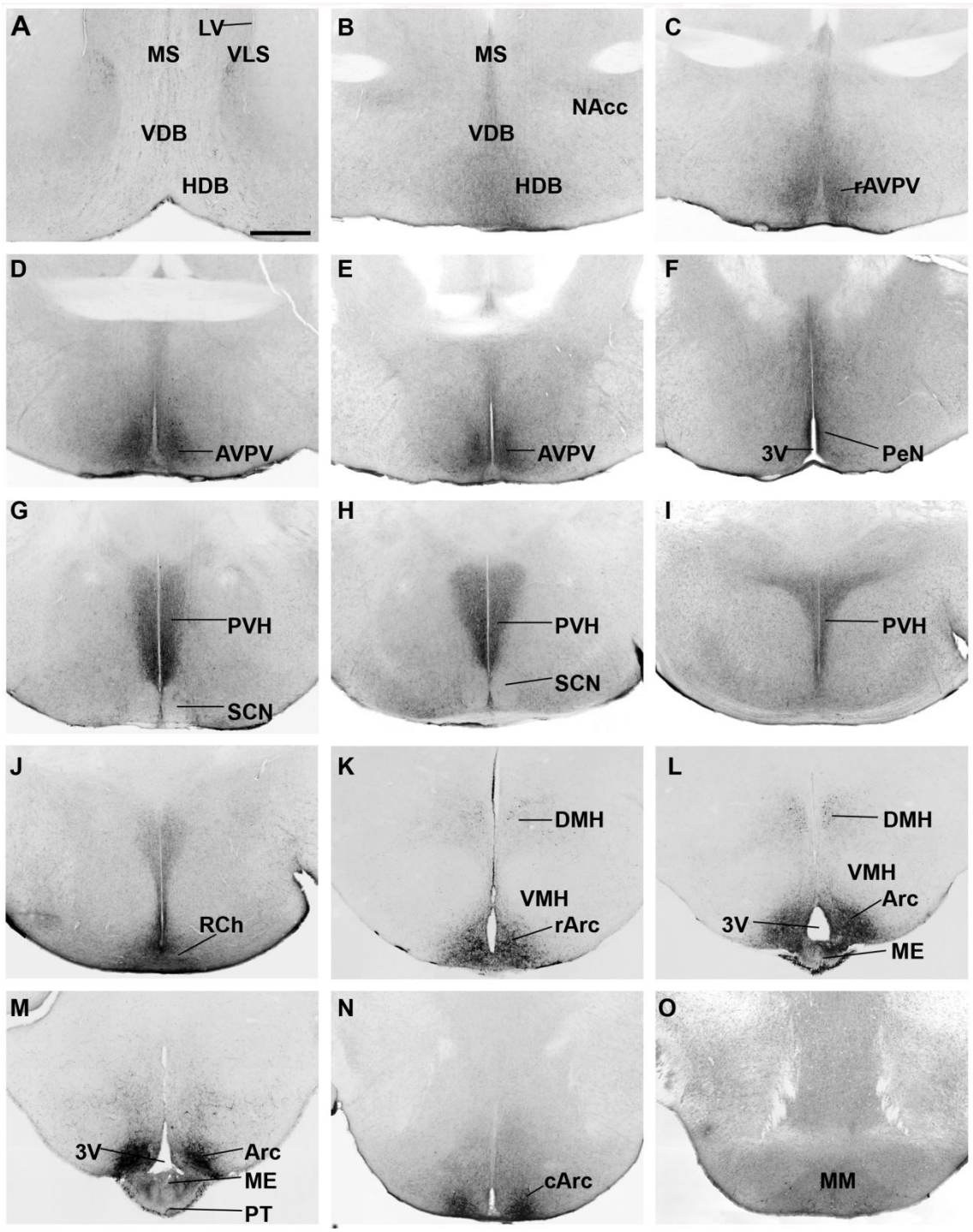


Plate 3.5: Low magnification (x40) brightfield photomicrographs of representative coronal sections (A-O; rostral to caudal) showing kisspeptin-immunoreactivity in the brain of a reproductive female naked mole-rat. 3V, third ventricle; AC, anterior commissure; Arc, arcuate nucleus; AVPV, anteroventral periventricular nucleus; cArc, caudal arcuate nucleus; DMH, dorsomedial hypothalamic nucleus; HDB, horizontal limb of the diagonal band; LV, lateral ventricle; ME, median eminence; MM, medial mammillary nucleus; MS, medial septum; NAcc, nucleus accumbens; PeN, periventricular nucleus; PVH, paraventricular hypothalamic nucleus; PT, pars tuberalis; rArc, rostral arcuate nucleus; rAVPV, rostral anteroventral periventricular nucleus; RCh, retrochiasmatic area; SCN, suprachiasmatic nucleus; VDB, vertical limb of the

diagonal band; VLS, ventrolateral septum; VMH, ventromedial hypothalamic nucleus. Scale bar = 500µm in A (applies to A-O).

Distribution of kisspeptin-ir processes

Most rostrally, kisspeptin-ir processes were found in the MS, VDB, HDB, the VLS and the NAcc (Plates 3.5A-B & 3.6A-B). In the MS, long bundles of parallel fibres appeared to stream dorsoventrally close to the midline (Plate 3.5B). More ventrally, kisspeptin-ir processes were distributed more laterally along the base of the hypothalamus (Plate 3.5B). Kisspeptin-ir processes were abundant in the vicinity of kisspeptin-ir cell bodies in the RP3V and PVH (Plates 3.5C-I & 3.6C-G). In the region of the RP3V, abundant kisspeptin-ir processes were distributed within and laterally to the vicinity of the kisspeptin-ir cell bodies (Plate 3.5D-F). Numerous kisspeptin-ir processes were running parallel to the wall of the 3V along the midline of the brain (Plate 3.5F). In the region of the PVH, kisspeptin-ir processes were largely contained within the nucleus, (Plates 3.5G-I & 3.6F-G) as revealed by a Nissl stain (Plate 3.6H & inset). Kisspeptin-ir processes in the PVH formed a distinct inverse triangular shape (Plate 3.5G-H). More caudally, the kisspeptin-ir processes thinned out dorsally, leaving an absence of immunoreactivity ventromediallly (Plate 3.5I). Although there was an almost complete absence of kisspeptin-ir processes from the SCN (Plates 3.5H & 3.6F) and VMH (Plate 3.5K), such processes were abundant in the RCh (Plate 3.5J), and leading caudally into the Arc and ME where the highest density of kisspeptin-ir processes were found (Plates 3.5K-N & 3.6K-L). Kisspeptin-ir processes were contained within the Arc and there were no long parallel bundles of kisspeptin-ir processes were present close of the midline of the brain at this level (Plate 3.5K-M). In contrast, there was only a sparse scattering of kisspeptin-ir processes in the DMH surrounding the kisspeptin-ir cell bodies (Plates 3.5K-N & 3.6J). Within the ME, kisspeptin-ir processes were distributed across its breath (Plates 3.5M & 3.6K). There were a few scattered kisspeptin-ir processes in the MM (Plate 3.6M). There were no differences in the distribution of kisspeptin-ir cell bodies between any of the eight animal groups.

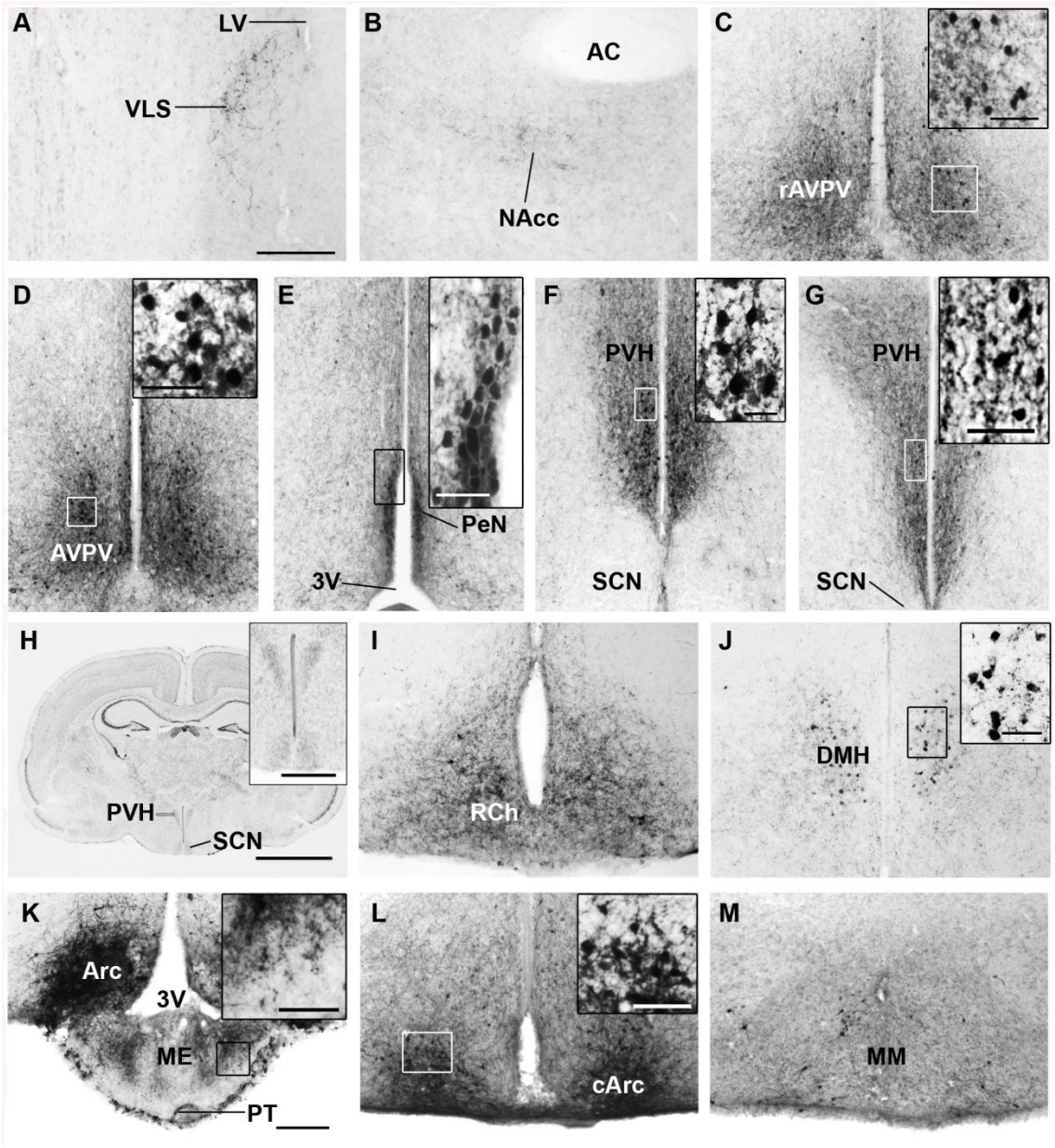


Plate 3.6: High magnification (x100) brightfield photomicrographs of representative coronal sections (A-M; rostral to caudal) showing kisspeptin-immunoreactivity in the brain of a reproductive female naked mole-rat showing kisspeptin immunoreactivity (A-G, I-M); with a Nissl-stained section (H) identifying the PVH at the same rostrocaudal level as shown for kisspeptin immunoreactivity (in G). Boxed regions are shown at higher magnification by insets within the same photomicrograph. 3V, third ventricle; AC, anterior commissure; Arc, arcuate nucleus; AVPV, anteroventral periventricular nucleus; cArc, caudal arcuate nucleus; DMH, dorsomedial hypothalamic nucleus; LV, lateral ventricle; ME, median eminence; MM, medial mammillary nucleus; NAcc, nucleus accumbens; PeN, periventricular nucleus; PVH, paraventricular hypothalamic nucleus; PT, pars tuberalis; rAVPV, rostral anteroventral periventricular nucleus; RCh, retrochiasmatic area; SCN, suprachiasmatic nucleus; VLS, ventrolateral septum. Scale bars = 200 µm in A (applies to A-G & I-M); 2 mm in H; 50 µm in the insets for C and L; 20 µm in the inset for D, E and F; 40µm in the insets in G, K and J, 500µm in the inset for H.

Number of kisspeptin-ir cell bodies in the RP3V

There was a large variation in the number of kisspeptin-ir cell bodies in the RP3V in the brains of all eight animal groups studied (Figure 3.3). The numbers of RP3V kisspeptin-ir cell bodies in breeding/reproductively-activated animals (groups 1-6) ranged from 28 to 1604 whilst the numbers of kisspeptin-ir cell bodies in subordinate animals (groups 7-8) ranged from 88 to 736. One-way ANOVA revealed a significant effect of animal group on the number of kisspeptin-ir cell bodies in the RP3V; $F(7,39)= 4.405$, $p= 0.001$. Planned contrasts indicated that reproductively-activated males (group 4) had significantly greater number of kisspeptin-ir cell bodies in the RP3V than male GDX breeders (group 6, $p= 0.042$), than female subordinates (group 7, $p= 0.012$) and than male subordinates (group 8, $p= 0.002$).

Breeding/reproductively-activated animals (groups 1-6) had significantly more kisspeptin-ir cell bodies in the RP3V than subordinate animals (groups 7-8); $t(45)= 3.706$, $p= 0.001$. There was no significant difference in the number of kisspeptin-ir cell bodies in the RP3V between breeders (groups 1-2) and reproductively-activated animals (groups 3-4); $t(16)= 1.695$, $p= 0.109$. There was no significant difference in the number of kisspeptin-ir cell bodies in the RP3V between intact breeders (groups 1-2) and GDX breeders (groups 5-6); $t(17)= 0.429$, $p= 0.673$. There was no significant difference in the number of kisspeptin-ir cell bodies in the RP3V between females (groups 1, 3, 5 and 7) and males (groups 2, 4, 6 and 8); $t(45)= 1.304$, $p= 0.199$. There was no significant correlation between body mass and number of kisspeptin-ir cell bodies in the RP3V (Figure 3.4); $r= 0.740$, $p= 0.632$.

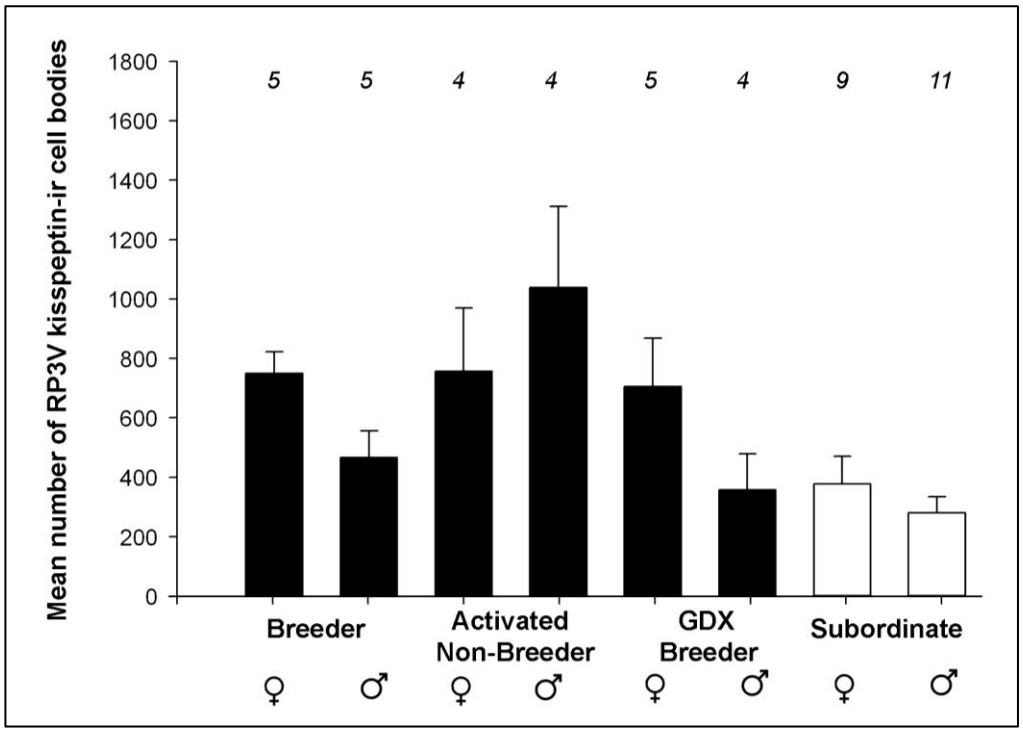


Figure 3.3: Mean (± SEM) number of kisspeptin-ir cell bodies in the RP3V of breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and male) and subordinates (female and male). Number of animals per group is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; open bars represent subordinate groups.

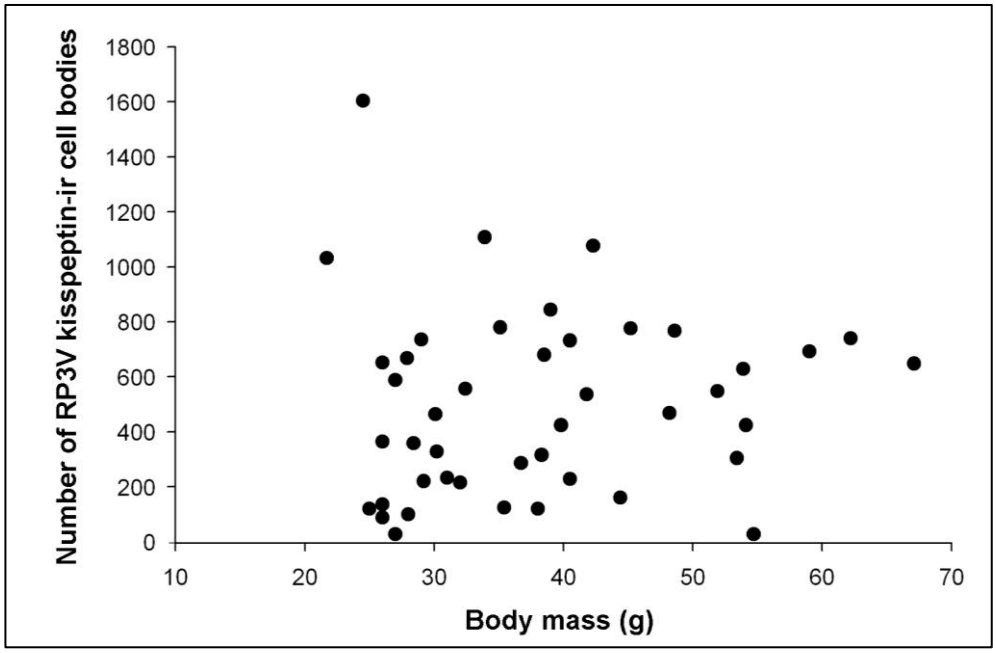


Figure 3.4: Lack of significant correlation between body mass (g) and total number of kisspeptin-ir cell bodies in the RP3V.

Number of kisspeptin-ir cell bodies in the PVH

There was a large variation in the number of kisspeptin-ir cell bodies in the PVH in the brains of all eight animal groups studied (Figure 3.5). The number of RP3V kisspeptin-ir cell bodies in breeding/reproductively-activated animals (groups 1-6) ranged from 32 to 740 whilst the number of kisspeptin-ir cell bodies in subordinate animals (groups 7-8) ranged from 36 to 628. One-way ANOVA revealed a lack of significant effect of animal group on the number of kisspeptin-ir cell bodies in the PVH; $F(7,39)= 1.920$, $p= 0.092$.

Breeding/reproductively-activated animals (groups 1-6) had significantly more kisspeptin-ir cell bodies in the PVH than subordinate animals (groups 7-8); $t(45)= 2.515$, $p= 0.016$. There was no significant difference in the number of kisspeptin-ir cell bodies in the PVH between breeding animals (groups 1-2) and reproductively-activated animals (groups 3-4); $t(16)= 0.933$, $p= 0.364$. There was no significant difference in the number of kisspeptin-ir cell bodies in the PVH between breeders (groups 1-2) and GDX breeders (groups 3-4); $t(17)= 1.451$, $p=0.165$. There was no significant difference in the number of kisspeptin-ir cell bodies in the PVH between females (groups 1, 3, 5 and 7) and males (groups 2, 4, 6 and 8); $t(45)= 0.977$, $p= 0.334$. There was no significant correlation between body mass and number of kisspeptin-ir cell bodies in the PVH (Figure 3.6); $r= 0.109$, $p= 0.480$.

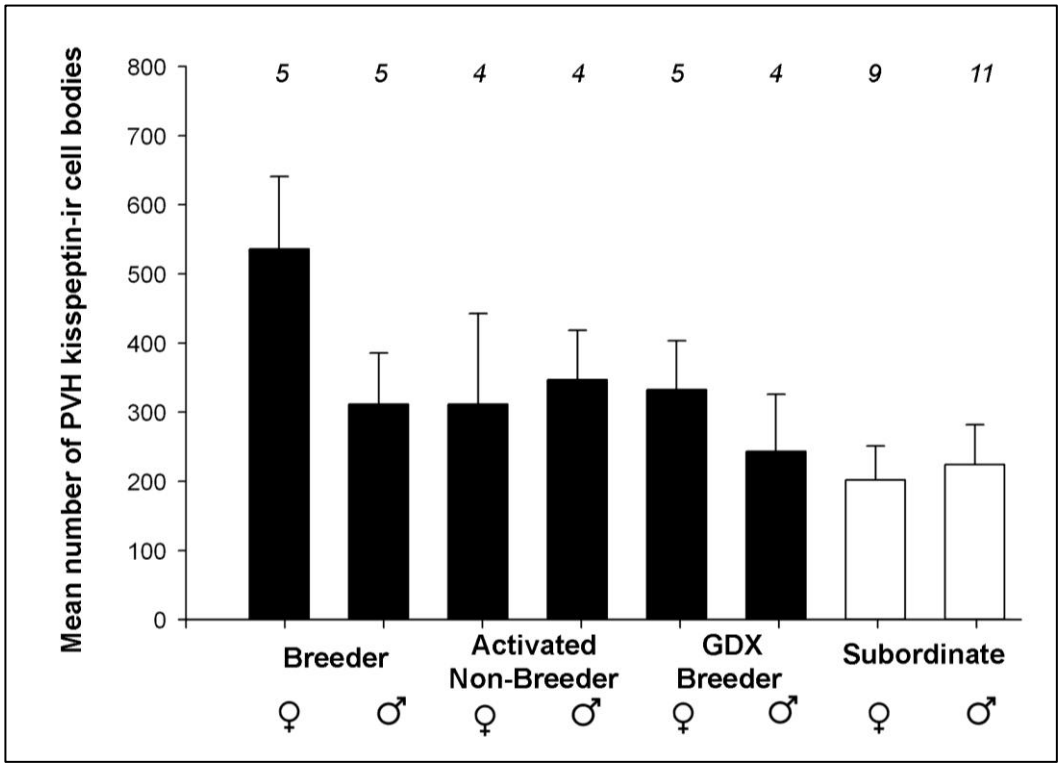


Figure 3.5: Mean (± SEM) number of kisspeptin-ir cell bodies in the PVH of breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and male) and subordinates (female and male). Number of animals per group

is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; open bars represent subordinate groups.

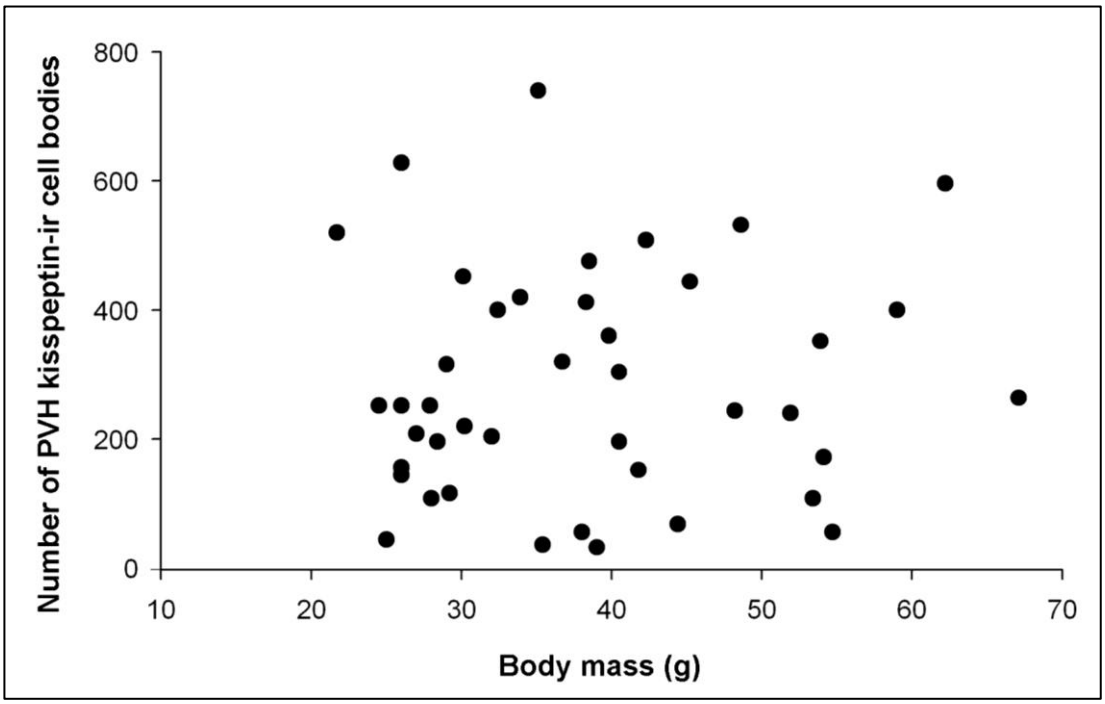


Figure 3.6: Lack of significant correlation between body mass (g) and total number of kisspeptin-ir cell bodies in the PVH.

Number of kisspeptin-ir cell bodies in the cArc

One-way ANOVA revealed no significant effect of animal group on the number of kisspeptin cell bodies in the cArc (Figure 3.7); $F(7,28)= 1.853$, $p= 0.116$. There was no significant difference in the number of kisspeptin-ir cell bodies in the cArc between breeding/reproductively-activated animals (groups 1-6) and subordinate animals (groups 7-8); $t(34)= 2.014$, $p= 0.052$. There was no significant difference in the number of kisspeptin-ir cell bodies in the cArc between breeders (groups 1-2) and reproductively-activated animals (groups 3-4); $t(13)=0.553$, $p= 0.590$. There was no significant difference in the number of kisspeptin-ir cell bodies in the cArc between breeders (groups 1-2) and GDX breeders (groups 5-6); $t(14)= 0.141$, $p= 0.890$. There was no significant difference in the number of kisspeptin-ir cell bodies in the cArc between females (groups 1, 3, 5 and 7) and males (groups 2, 4, 6 and 8); $t(34)= 1.865$, $p=0.071$. There was no significant correlation between body mass and the number of kisspeptin-ir cell bodies in the cArc; $r= 0.104$, $p= 0.566$.

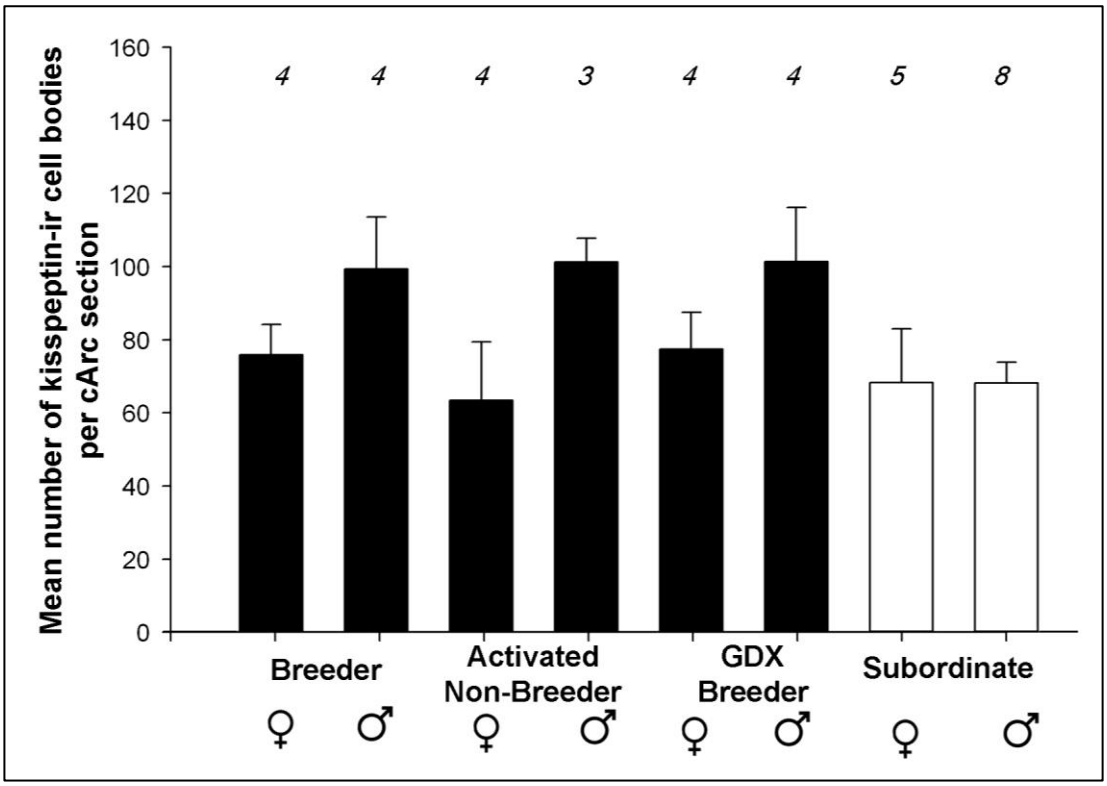


Figure 3.7: Mean (± SEM) number of kisspeptin-ir cell bodies per caudal arcuate nucleus (cArc) section in breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and males) and subordinates (female and male). Number of animals per group is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; open bars represent subordinate groups.

Number of kisspeptin-ir cell bodies in the DMH

One-way ANOVA revealed no significant effect of animal group on the number of kisspeptin cell bodies in the DMH (Figure 3.8); $F(7,30)= 1.811$, $p= 0.122$. There was no significant difference in the number of kisspeptin-ir cell bodies in the DMH between breeding/reproductively-activated animals (groups 1-6) and subordinate animals (groups 7-8); $t(36)= 1.922$, $p= 0.630$. There was no significant difference in the number of kisspeptin-ir cell bodies in the DMH between breeders (groups 1-2) and reproductively-activated animals (groups 3-4); $t(14)= 1.130$, $p= 0.277$. There was no significant difference in the number of kisspeptin-ir cell bodies in the DMH between breeders (groups 1-2) and GDX (breeders 5-6); $t(15)= 0.747$, $p= 0.466$. Females (groups 1, 3, 5 and 7) had significantly more kisspeptin-ir cell bodies in the DMH than males (groups 2, 4, 6 and 8); $t(36)= 2.197$, $p= 0.035$. There was no significant correlation between body mass and the number of kisspeptin-ir cell bodies in the DMH; $r= 0.190$, $p= 0.268$.

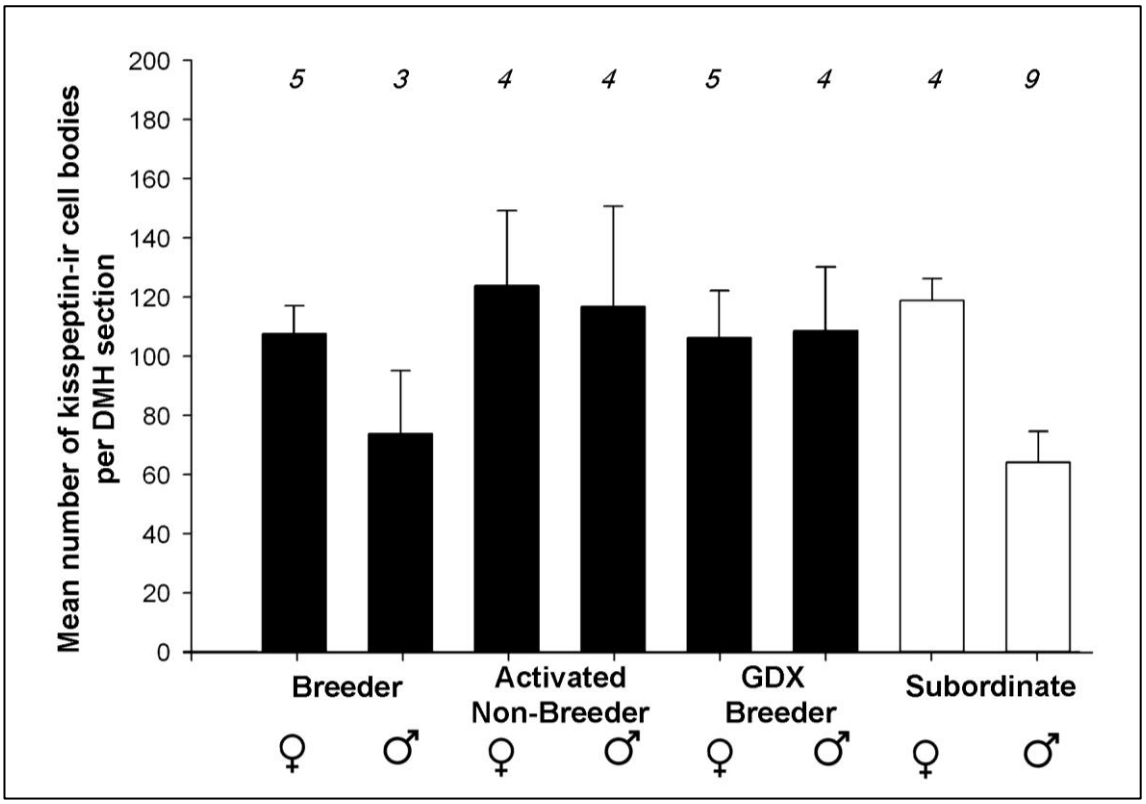


Figure 3.8: Mean (± SEM) number of kisspeptin-ir cell bodies per dorsomedial hypothalamic nucleus (DMH) section in breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and males) and subordinates (female and male). Number of animals per group is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; white bars represent subordinate groups.

Relationship between GnRH-1- and kisspeptin-immunoreactivity

Correlations between the number of GnRH-1- and kisspeptin-ir cell bodies in each individual were determined using animals from all the 8 groups. There was a significant positive correlation between the number of GnRH-1-ir cell bodies and the number of kisspeptin-ir cell bodies in the RP3V (Fig. 3.9); r= 0.365, p= 0.027. There was a significant positive correlation between the number of GnRH-1-ir cell bodies and the number of kisspeptin-ir cell bodies in the PVH (Fig. 3.10); r= 0.361, p= 0.028. There was no significant correlation between the number of GnRH-1-ir cell bodies and the number of kisspeptin-ir cell bodies in the cArc (Fig. 3.11); r= -0.082, p= 0.657. There was no significant correlation between the number of GnRH-1-ir cell bodies and the number of kisspeptin-ir cell bodies in the DMH (Fig. 3.12); r= -0.086, p= 0.633.

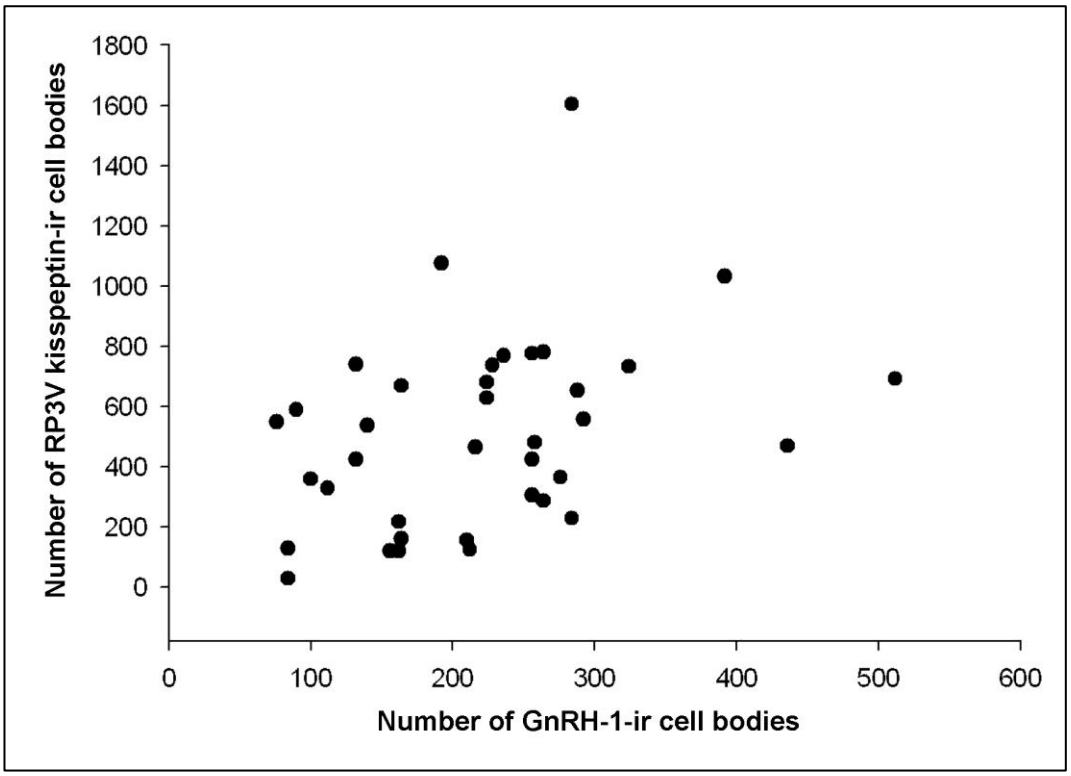


Figure 3.9: Significant correlation between number of GnRH-1-ir cell bodies and number of kisspeptin-ir cell bodies in the RP3V.

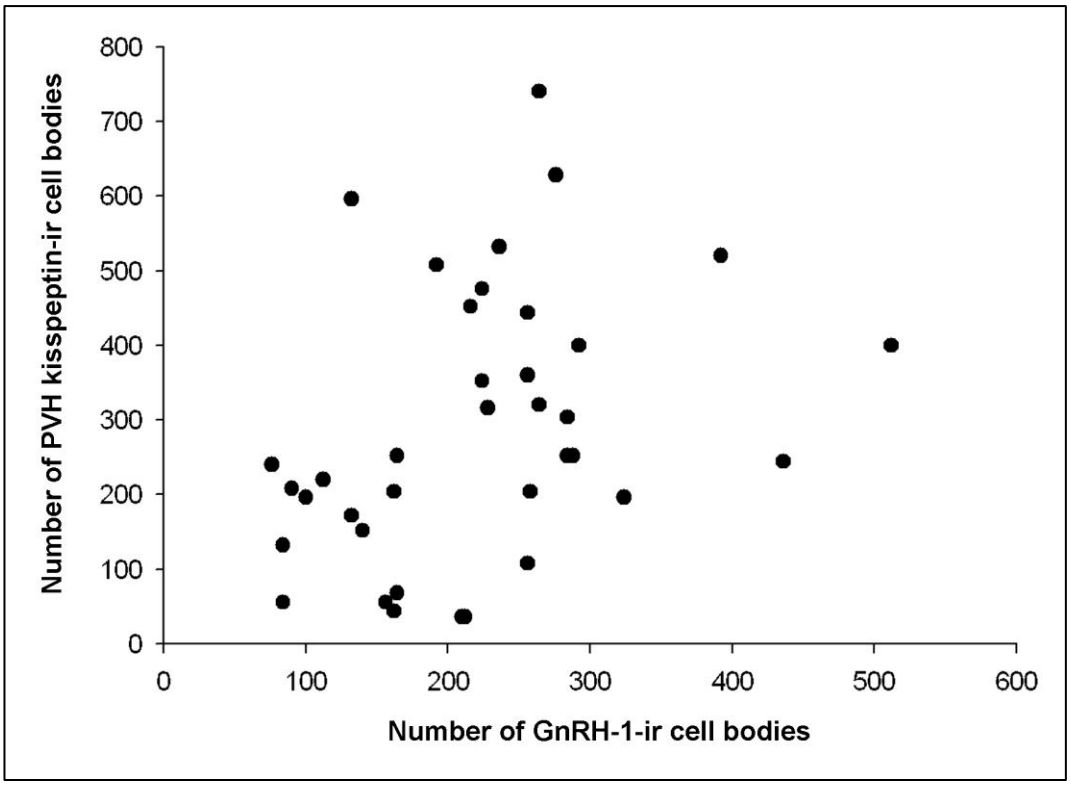


Figure 3.10: Significant correlation between number of GnRH-1-ir cell bodies and number of kisspeptin-ir cell bodies in the PVH.

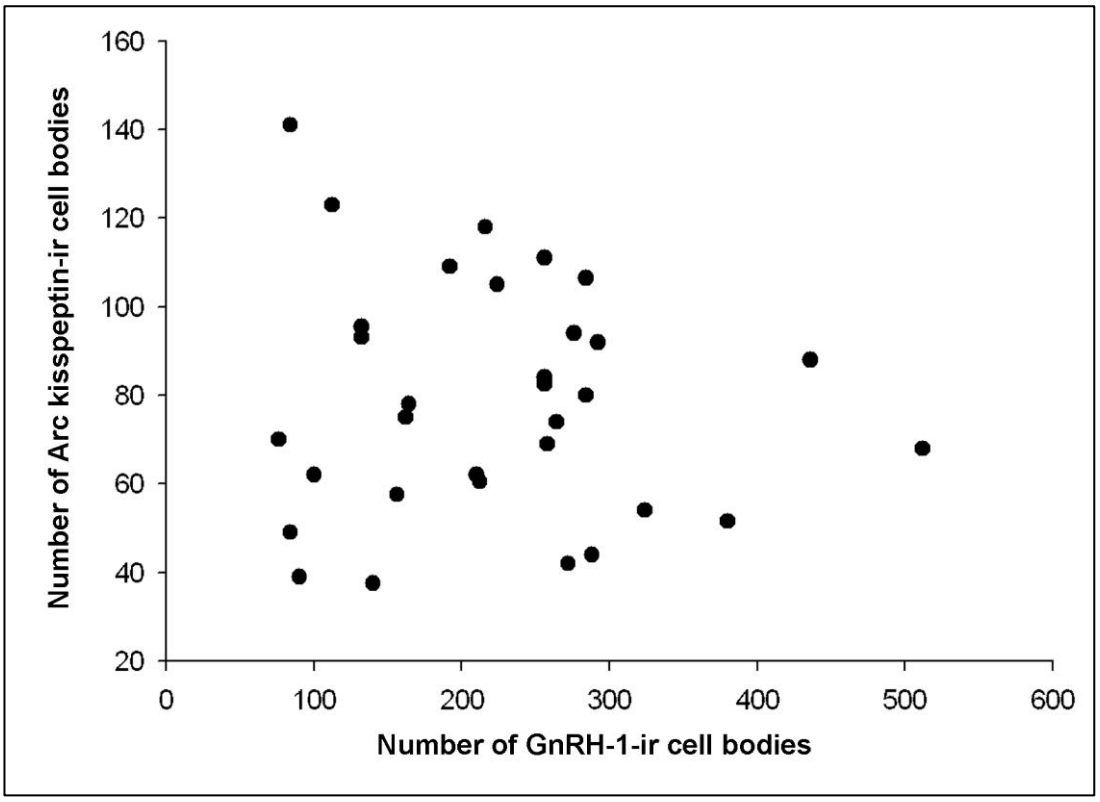


Figure 3.11: Lack of significant correlation between number of GnRH-1-ir cell bodies and number of kisspeptin-ir cell bodies in the Arc.

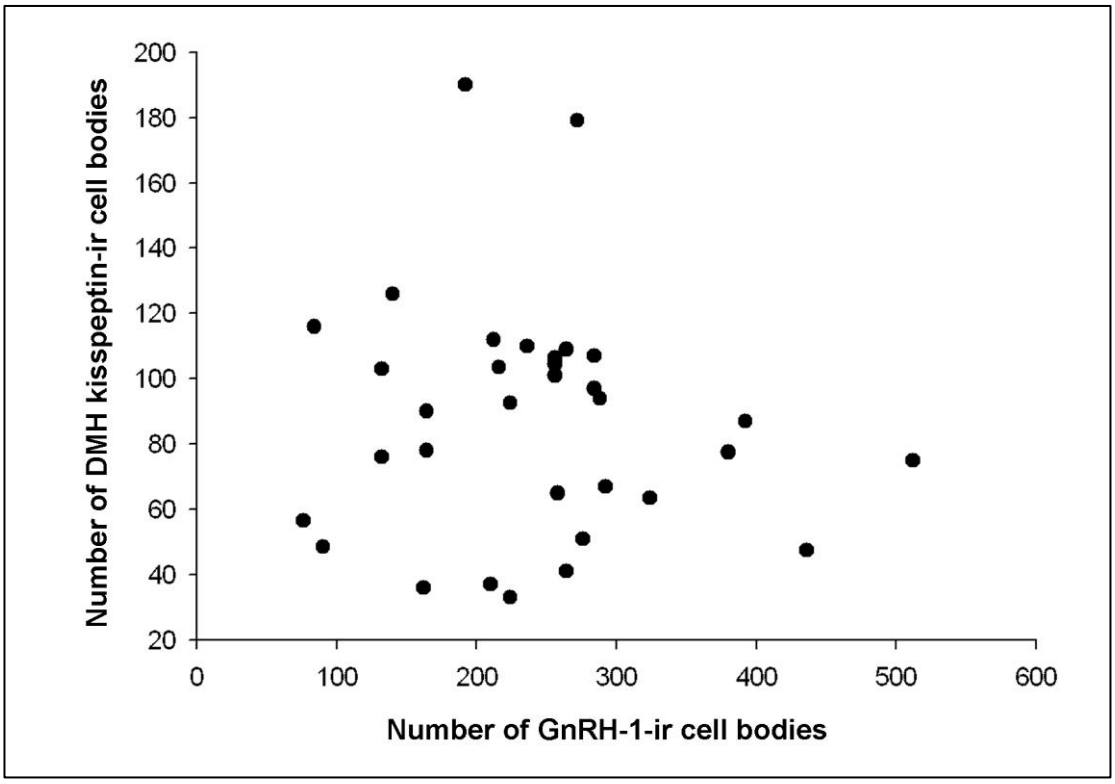


Figure 3.12: Lack of significant correlation between number of GnRH-1-ir cell bodies and number of kisspeptin-ir cell bodies in the DMH.

Table 3.3: Mean (± SEM) plasma testosterone, oestradiol and progesterone

	Animal group	Sex	Testosterone ng/ml	Oestradiol ng/ml	Progesterone ng/ml
1	Breeder	F	0.12 ± 0.01 ^{A}	0.07 ± 0.04	13.79 ± 3.74 ^{A}
2	Breeder	M	7.90 ± 1.31 ^{B}	0.002 ± 0.002	2.98 ± 0.53 ^{B}
3	Activated non-breeder	F	0.13 ± 0.03 ^{A}	0.02 ± 0.01	6.65 ± 4.30
4	Activated non-breeder	M	6.85 ± 2.45 ^{B}	0.02 ± 0.02	3.84 ± 0.79
5	GDX breeder	F	0.23 ± 0.15 ^{A}	0.27 ± 0.19	1.73 ± 0.46 ^{B}
6	GDX breeder	M	0.11 ± 0.002 ^{A}	0.09 ± 0.05	2.56 ± 0.56 ^{B}
7	Subordinate	F	0.28 ± 0.09 ^{A}	0.15 ± 0.09	3.03 ± 0.44
8	Subordinate	M	2.39 ± 1.59	0.00 ± 0.00	6.07 ± 2.36

For each hormone, statistically significant differences ($p < 0.05$) exist where the superscript letters differ.

Plasma testosterone levels

All female animals (groups 1, 3, 5 and 7) had very low levels of plasma testosterone (Table 3.3, Figure 3.13). One-way ANOVA revealed a significant effect of animal group on the level of plasma testosterone; $F(7,35)= 8.293$, $p= 0.000$. Planned contrasts indicated that male breeders (group 2) had significantly higher plasma testosterone levels than females breeders (group 1; $p= 0.000$), than female reproductively-activated animals (group 3; $p= 0.000$), than female GDX breeders (group 5; $p= 0.001$), than male GDX breeders (group 6; $p= 0.001$) and higher than female subordinates (group 7; $p= 0.001$). Furthermore, reproductively-activated males (group 4) had significantly higher plasma testosterone levels than females breeders (group 1; $p= 0.003$), than reproductively-activated females (group 3, $p= 0.003$), than female GDX breeders (group 5, $p= 0.007$), than male GDX breeders (group 6; $p= 0.006$), and higher than female subordinates (group 7; $p= 0.007$). In other words, both the male breeders (group 2) and reproductively-activated males (group 4) have significantly higher plasma testosterone levels than all other female groups (groups 1, 3, 5 and 7) and GDX male breeders (group 6).

There was no significant correlation between plasma testosterone level and the number of kisspeptin-ir cell bodies in the RP3V; $r= 0.200$, $p= 0.264$. There was no significant correlation between plasma testosterone level and the number of kisspeptin-ir cell bodies in the PVH; $r= -0.108$, $p= 0.550$. There was no significant correlation between plasma testosterone level and the number of kisspeptin-ir cell bodies in the cArc; $r= 0.302$, $p= 0.111$. There was no significant correlation between plasma testosterone level and the number of kisspeptin-ir cell bodies in the DMH; $r= 0.179$, $p= 0.336$.

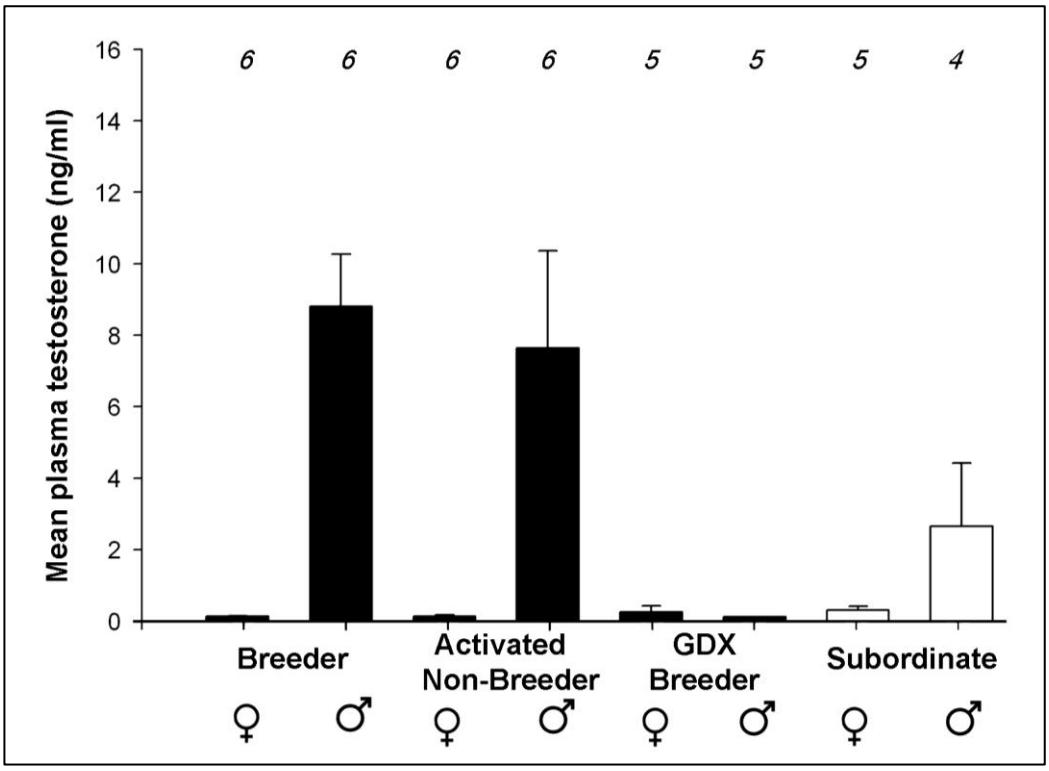


Figure 3.13: Mean (± SEM) plasma testosterone (ng/ml) in breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and males) and subordinates (female and male). Number of animals per group is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; open bars represent subordinate groups.

Plasma and adrenal oestradiol levels

Plasma oestradiol levels were generally low in all animal groups (Table 3.3, Figure 3.14). One-way ANOVA revealed a lack of significant effect of animal group on the plasma oestradiol level. Unexpectedly, gonadectomy did not reduce plasma oestradiol levels in females, in fact, the female GDX breeders (group 5) had the highest plasma oestradiol concentration (0.98 ng/ml). This anomaly may be explained by the relatively high oestradiol level found in the adrenals of female GDX breeders (group 5; Table 3.4). One-way ANOVA revealed a significant effect of group on the adrenal oestradiol levels; $F(7,13)= 16.576$, $p= 0.000$. Planned contrasts indicated that female GDX breeders (group 5) had significantly higher adrenal oestradiol levels than all other animals groups (Table 3.4). One-way ANOVA revealed a lack of significant effect of group on adrenal weight; $F(7,13)= 1.442$, $p= 0.270$.

There was a lack of significant correlation between the plasma oestradiol level and the number of kisspeptin-ir cell bodies in the RP3V; $r= 0.043$, $p= 0.811$. There was a lack of significant correlation between the plasma oestradiol level and the number of

kisspeptin-ir cell bodies in the PVH; r= 0.002, p= 0.992. There was a lack of significant correlation between the plasma oestradiol level and the number of kisspeptin-ir cell bodies in the cArc; r= -0.010 and p= 0.959. There was a lack of significant correlation between the plasma oestradiol level and the number of kisspeptin-ir cell bodies in the DMH; r= -0.023, p= 0.901. There was a significant positive correlation between plasma oestradiol levels and adrenal oestradiol levels; r= 0.787, p= 0.000. There was a significant negative correlation between adrenal weight and adrenal oestradiol levels; r= -0.439, p= 0.047. There was no significant correlation between adrenal weight and body weight; r= 0.119, p= 0.617.

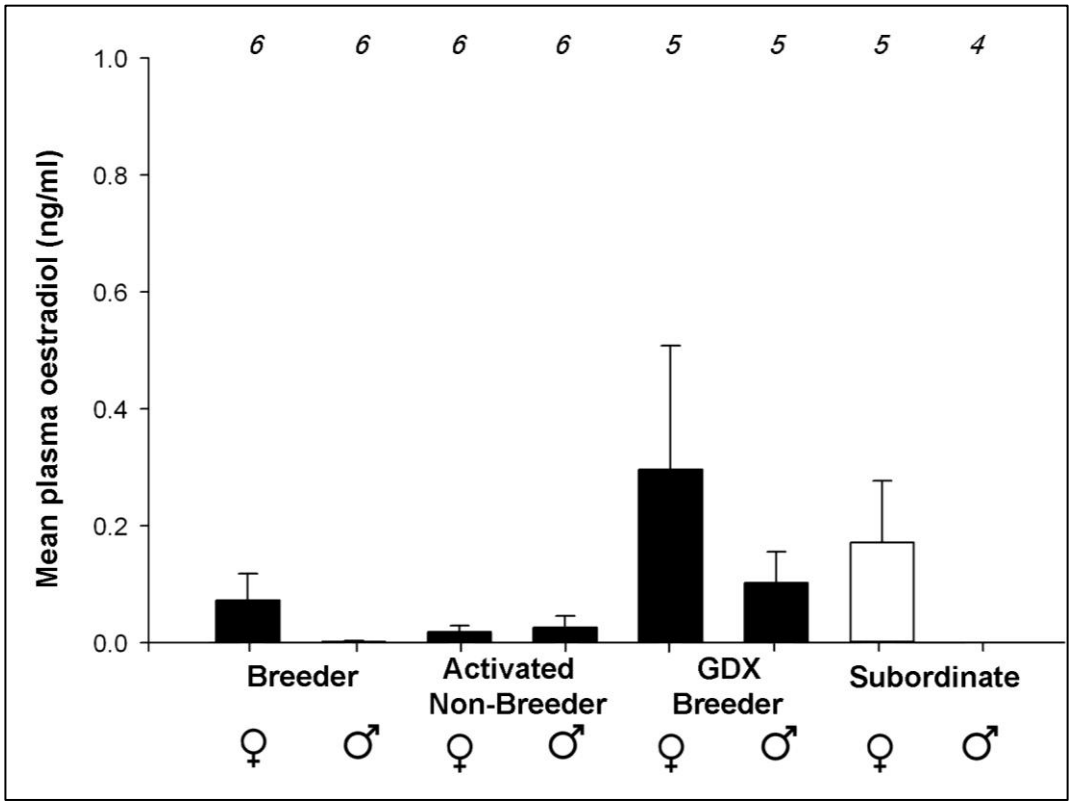


Figure 3.14: Mean (± SEM) plasma oestradiol (ng/ml) in breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and males) and subordinates (female and male). Number of animals per group is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; open bars represent subordinate groups.

Table 3.4: Mean (± SEM) adrenal oestradiol level and adrenal mass.

	Animal group	Sex	N	Adrenal oestradiol pg/mg	Adrenal mass mg
1	Breeder	F	3	2.19 ± 0.38 ^{A}	13.67 ± 2.85
2	Breeder	M	3	2.12 ± 0.23 ^{A}	14.00 ± 3.06
3	Activated non-breeder	F	2	2.09 ± 0.13 ^{A}	17.00 ± 2.00
4	Activated non-breeder	M	2	2.27 ± 0.45 ^{A}	15.50 ± 3.50
5	GDX breeder	F	2	7.42 ± 0.57 ^{B}	12.50 ± 6.50
6	GDX breeder	M	2	1.47 ± 0.45 ^{A}	28.00 ± 0.00
7	Subordinate	F	3	2.71 ± 0.79 ^{A}	17.67 ± 5.24
8	Subordinate	M	4	1.26 ± 0.07 ^{A}	24.75 ± 5.30

Statistically significant differences ($p < 0.05$) exist where the superscript letters differ.

Plasma progesterone levels

Breeding females (group 1) had the highest plasma progesterone levels out of all the groups (Table 3.3, Figure 3.15). One-way ANOVA revealed a significant effect of animal group on plasma progesterone levels; $F(7,34) = 2.773$, $p = 0.022$. Planned contrasts indicated that breeding females (group 1) had significantly higher plasma progesterone levels than reproductively-activated females (group 2; $p = 0.040$), higher than female GDX breeders (group 5; $p = 0.024$) and higher than male GDX breeders (group 6; $p = 0.043$).

There was no significant correlation between plasma progesterone levels and the number of kisspeptin-ir cells in the RP3V; $r = 0.206$, $p = 0.250$. There was a significant positive correlation between plasma progesterone levels and the number of kisspeptin-ir cell bodies in the PVH; $r = 0.353$, $p = 0.044$. There was no significant correlation between plasma progesterone levels and the number of kisspeptin-ir cell bodies in the cArc; $r = 0.122$, $p = 0.530$. There was no significant correlation between plasma progesterone levels and the number of kisspeptin-ir cell bodies in the DMH; $r = 0.052$, $p = 0.780$. The implications of these results are discussed further below.

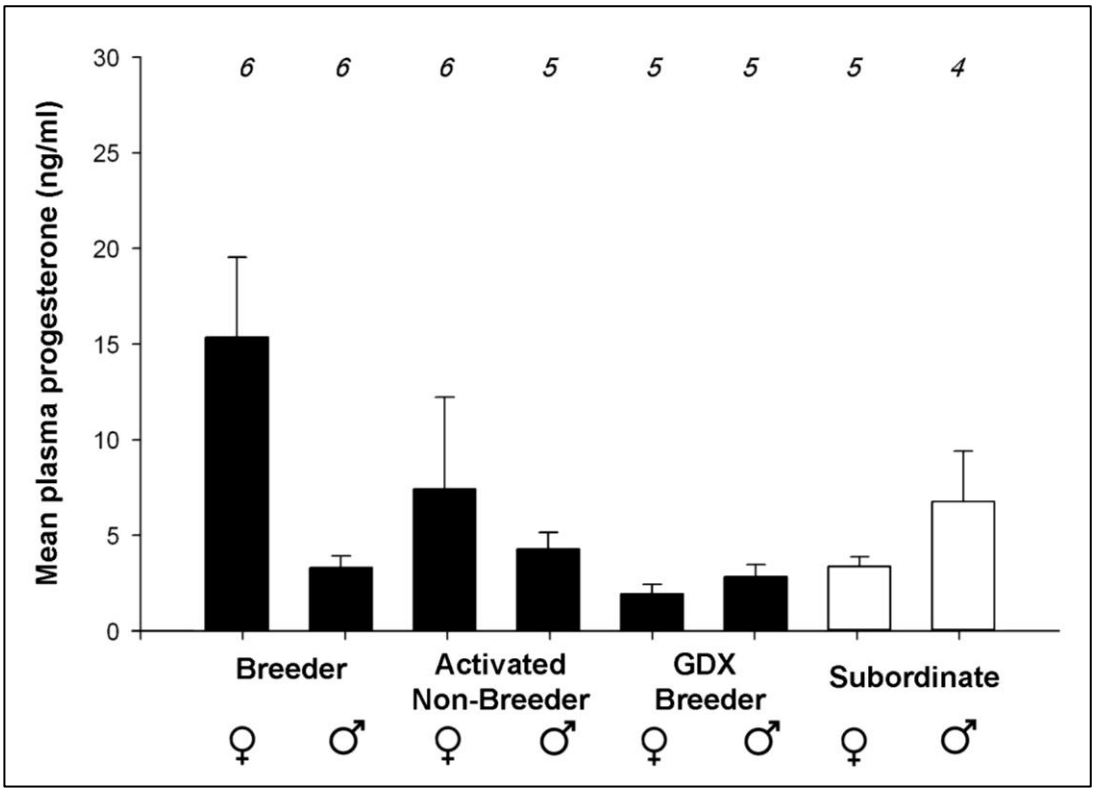


Figure 3.15: Mean (± SEM) plasma progesterone (ng/ml) in breeders (female and male), reproductively-activated non-breeders (female and male), gonadectomised (GDX) breeders (female and males) and subordinates (female and male). Number of animals per group is noted at the top in italics. ♀ = female animal group; ♂ = male animal group. Black bars represent breeding/reproductively-activated groups; open bars represent subordinate groups.

DISCUSSION

Naked mole-rats provide an extreme example of socially suppressed fertility and cooperative breeding, where reproduction is restricted to the socially dominant queen and her male consorts. In subordinates of both sexes, the physiological processes required for reproduction are inhibited by the presence of the queen. Thus, during their long adult lifetime, most females and males experience little or no gonadal maturation (Jarvis, 1991). Various changes occur in endocrine and neuroanatomical parameters after subordinates have been released from the dominant presence of the queen (Faulkes *et al.*, 1990a; Faulkes *et al.*, 1990b; Faulkes and Abbott, 1991; Faulkes *et al.*, 1991; Holmes *et al.*, 2007; Holmes *et al.*, 2011). In recent years, extensive research on mice and rats has elucidated the contributions made by hypothalamic kisspeptin neurones to the onset of puberty and maintenance of fertility; these include activation of GnRH-1 neurones and mediation of oestradiol's stimulatory effects on those neurones (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Messager *et al.*, 2005; Navarro *et al.*, 2005). In the present study, an immunohistochemical approach was used to chart and quantify the GnRH-1 and kisspeptin systems in naked mole-rats that had remained subordinate, those who became reproductively-activated, and those who became reproductively-activated and produced offspring, with or without subsequent gonadectomy.

Specificity of the immunoreactivity

The successful neutralisation of the GnRH-1 and kisspeptin antibodies with their respective peptides indicates that both of these antibodies are specific. Significance of these neutralisation tests are discussed further below for each anatomical region.

The distribution of GnRH-1-ir cell bodies

GnRH-1-ir cell bodies are distributed in a loose continuum along the septo-preoptico-hypothalamo pathway in naked mole-rats, with a few isolated cells located caudal to the SCN. This distribution is similar to the GnRH-1 distribution in other rodents, such as rats (Coen *et al.*, 1990); mice (Urbanski, 1991); Syrian hamsters, *Mesocricetus auratus,* (Urbanski *et al.*, 1991; Richardson *et al.*, 1999), musk shrews, *Suncus murinus (*Dellovade *et al.*, 1995a; Dellovade *et al.*, 1995b) and prairie voles, *Microtus ochrogaster* (Kriegsfeld *et al.*, 2000). Previous studies have also implicated a comparable GnRH-1 distribution to other mole-rats, such as Damaraland mole-rats (Molteno *et al.*, 2004); Cape dune mole-rats, *Bathyergus suillus* (Hart *et al.*, 2008); common mole-rats, *Cryptomys hottentotus* (Du Toit *et al.*, 2006); highveld mole-rats, *Cryptomys hottentotus pretoriae* (Du Toit *et al.*, 2006); Cape mole-rats, *Georychus*

capensis, (Oosthuizen *et al.*, 2008) and Natal mole-rats, *Hottentotus natalensis* (Oosthuizen *et al.*, 2008).

However, there are inter-species differences in the distribution of GnRH-1 cell bodies, and these differences may be dependent upon the extent of their caudal migration from the olfactory placode (Schwanzel-Fukuda and Pfaff, 1989). For example, in minks, the majority (80%) of GnRH-1 neurones migrate into the MBH (Ntoumi *et al.*, 1994). In contrast, there were very few GnRH-1 cell bodies in the MBH of rats (Witkin *et al.*, 1982; King and Anthony, 1984; Merchenthaler *et al.*, 1984). Furthermore, GnRH-1 neurones rarely migrated past the POA of opossums, with most GnRH-1 cell bodies located in the MS, HDB and VDB (Schwanzel-Fukuda *et al.*, 1988). Nevertheless, in most mammals where GnRH-1 cell bodies migrate into the MBH, these cells usually constitute a relatively small proportion of the total number of GnRH-1 cells (Silverman *et al.*, 1994). This is true of the naked mole-rats where only approximately 5% of the total number of GnRH-1-ir cell bodies are found caudal to the SCN in the MBH and RCh regions. The other 95% of GnRH-1-ir cell bodies are located rostral to the SCN in the MS, VDB, HDB, MPOA or OVLT region. In most mole-rat species investigated, the majority of GnRH-1-ir cell bodies are located in the MS/HDB/VDB/MPOA region: naked mole-rats: ~95%, Damaraland mole-rats: ~85%, Natal mole-rats: ~65%, Cape mole-rats: ~90%, Highveld mole-rats: ~81% (Molteno *et al.*, 2004; Du Toit *et al.*, 2006; Oosthuizen *et al.*, 2008). In contrast, GnRH-1-ir cells bodies are found in approximately similar proportions in the MS/HDB/VDB//MPOA and MBH of common and Cape dune mole-rats (Du Toit *et al.*, 2006; Hart *et al.*, 2008).

The results of this study indicate that the distribution of GnRH-1-ir cell bodies in all naked mole-rats is uniform amongst the eight animal groups tested. Similar uniform distributions have been observed in other mole-rat species, irrespective of sex or reproductive status (Molteno *et al.*, 2004; Du Toit *et al.*, 2006; Hart *et al.*, 2008; Oosthuizen *et al.*, 2008). These studies suggest that GnRH-1 neurone distributions are species specific and not influenced by environmental or social plasticity.

The distribution of GnRH-1-ir processes

GnRH-1-ir processes are present along the septo-preoptico-hypothalamo continuum from the MS/VDB/HDB, to the ME. As found in all mammalian species studied to date, two areas show particularly dense GnRH-1 immunoreactivity; the OVLT and the ME. Caudal to the OVLT, GnRH-1-ir processes are present within the vestigial OCh, the membranous structure which forms the floor of the 3V. The presence of GnRH-1-ir processes are also found under the fully developed optic chiasm in rats (Hoffman and Gibbs, 1982) and within the vestigial optic chiasm in Damaraland, Common, Highveld and Cape mole-rats, but not in Natal mole-rats (Molteno *et al.*, 2004;Du Toit *et al.*,

2006; Hart et al., 2008; Oosthuizen et al. 2008). GnRH-1-ir processes are absent from the SCN of naked mole-rats and all other species of mole-rats studied to date (Molteno *et al.*, 2004; Hart *et al.*, 2008; Oosthuizen *et al.*, 2008).

In the naked mole-rat, GnRH-1-ir cell processes are located in the ME where they form an extensive arching pattern from the borders of the 3V, achieving a dense aggregation throughout the ME. GnRH-ir processes are distributed across the breadth of the ME and a similar distribution of GnRH immunoreactivity is observed in the ME of Natal mole-rats (Oosthuizen *et al.*, 2008), common mole-rats (Du Toit *et al.*, 2006) and highveld mole-rats (Du Toit *et al.*, 2006). In contrast, GnRH-1-ir distribution is concentrated in lateral margins of the ME in Cape mole-rats (Oosthuizen *et al.*, 2008) and Damaraland mole-rats (Molteno *et al.*, 2004). GnRH-1-ir processes are concentrated in the external zone of the ME of Japanese quails (Mikami and Yamada, 1984). This distribution is different in bats whereby their GnRH-1-ir processes are concentrated in the internal zone of the ME (Fernandez *et al.*, 1992). Like naked mole-rats, intermediate conditions are found in humans, monkeys and ferrets, whereby considerable numbers of GnRH-1-ir processes are located in both the internal and external zones of the ME (Anthony *et al.*, 1984).

The density of GnRH-1-immunoreactivity in the ME is significantly higher in subordinate Damaraland mole-rats (Molteno *et al.*, 2004), Natal mole-rats (Oosthuizen *et al.*, 2008) and highveld mole-rats (Du Toit *et al.*, 2006). Indeed, a similar finding is observed in female white-footed mice, where there is a higher density of GnRH-1-ir processes in the ME during inhibitory, short photoperiods (Glass, 1986). Furthermore, studies on musk shrews show that mating behaviour that fails to induce ovulation is associated with a higher density of GnRH-1 immunoreactivity in the ME than that found after successful reflex ovulation (Dellovade *et al.*, 1995b). Since GnRH is transported from the site of synthesis in the cell body to the nerve terminals where it is released into the portal vessels in the ME, a high density of GnRH-1 immunoreactivity in the ME may indicate an inhibition of GnRH-1 release in subordinate mole-rats (Molteno *et al.*, 2004; Du Toit *et al.*, 2006; Oosthuizen *et al.*, 2008). This accumulation of GnRH-1 in the terminals of the ME may be one of the factors contributing to the suppression of the HPG axis in subordinate/non-reproductive animals.

Previous studies in naked mole-rats have shown that the LH responses of subordinates to exogenous GnRH-1 injections are reduced compared to the breeders, but still present (Faulkes *et al.*, 1990b; Faulkes *et al.*, 1991; Bennett *et al.*, 1993), indicating that reproductive suppression in subordinate naked mole-rats may be due to chronically reduced release of GnRH-1 from the hypothalamus. In this present study, there were no significant differences in the density of GnRH-1-ir processes in the ME between breeding and subordinate naked mole-rats of both sexes. This suggests that

there is no accumulation of GnRH-1 at the ME in subordinate naked mole-rats and that reproductive suppression may occur higher up at the level of kisspeptin rather than at the level of GnRH-1 release.

Number of GnRH-1-ir cell bodies

There are large variations in the number of GnRH-1-ir cells in the brains of all eight naked mole-rat groups studied. The total number of GnRH-1-ir cell bodies detected in the eight groups of naked mole-rats ranged from 76 to 588. This degree of heterogeneity is not unusual. Studies describing individual differences for this parameter have reported a four-fold range in ewes and springboks, irrespective of reproductive state (Lehman *et al.*, 1986; Robinson *et al.*, 1997). The mean number of GnRH-1-ir cell bodies across the naked mole-rat groups (235 ± 16 SEM) is lower than that found in Cape mole-rats (426 ± 35; Oosthuizen *et al.*, 2008), Damaraland mole-rats (648 ± 33; Molteno *et al.*, 2004), Common mole-rats (605 ± 60; Du Toit *et al.*, 2006), Natal mole-rats (721 ± 41; Oosthuizen *et al.*, 2008) Syrian hamsters (~400; Urbanski *et al.*, 1991) and mice (~600; Herbison *et al.*, 2008). The larger number in Highveld mole-rats (1489 ± 183; Du Toit *et al.*, 2006) is similar to that in Cape Dune mole-rats (~1000-1600; Hart *et al.*, 2008), rats (~1300; Wray and Hoffman, 1986), and sheep, springboks and rhesus monkeys (~1000–2000; Silverman *et al.*, 1982; Lehman *et al.*, 1986,Robinson *et al.*, 1997).

Consistent with previous studies on other mole-rat species (Molteno *et al.*, 2004; Du Toit *et al.*, 2006; Hart *et al.*, 2008; Oosthuizen *et al.*, 2008), the present findings show no significant differences in the number of GnRH-1-ir cell bodies in the naked mole-rat brain according breeding history, reproductive status, gonadectomy or sex. In other species of mole-rats, the number of GnRH-1-ir cell bodies is not regulated by social relations (Damaraland mole-rats and Natal mole-rats), by season (Cape mole-rats and Cape Dune mole-rats) or by both of these factors (Common mole-rats and Highveld mole-rats) (Molteno *et al.*, 2004; Du Toit *et al.*, 2006; Hart *et al.*, 2008; Oosthuizen *et al.*, 2008). The number of GnRH-1-ir cell bodies does not differ between the sexes in adult rats or springboks (Wray and Hoffman, 1986; Robinson *et al.*, 1997), between mid-luteal and anestrous ewes (Lehman *et al.*, 1986), between breeding and non-breeding male springboks (Robinson *et al.*, 1997) or between male Syrian hamsters under long or short day photoperiods (Urbanski *et al.*, 1991).

In naked mole-rats, gonadectomy did not lead to a significant change in the number of detectable GnRH-1-ir cell bodies in breeders of either sex. The maintenance of high numbers of GnRH-1 cell bodies in the GDX naked mole-rats is intriguing. There is limited information about whether removal of gonadal steroids affects the number of detectable GnRH-1-ir cell bodies in other species. No change was seen in ewes after

gonadectomy (Lehman *et al.*, 1986). In contrast, gonadectomy in rats has been reported to reduce their number in males, but not in females (Shivers *et al.*, 1983). Tonic gonadotrophin secretion in primates and rats is regulated by a negative feedback loop between the gonads and the HPG axis (Knobil, 1974; Shivers *et al.*, 1983), and interruption of this negative feedback loop by gonadectomy results in a marked increase in the levels of gonadotrophins (Yen and Tsai, 1971; Hodges, 1978). Further studies on the GnRH-1 system in naked mole-rats will help us determine the effects of gonadal steroids on the HPG axis.

Despite the lack of differences in numbers of GnRH-1-ir cell bodies between subordinate and reproductive naked mole-rats, significant changes in the GnRH-1 system overall have been observed in other mole-rats and rodents. For example, non-reproductive female highveld mole-rats display significantly larger GnRH-1-ir cell bodies than the reproductive females (Du Toit *et al.*, 2006). In the seasonally breeding Syrian hamster (*Mesocricetus auratus*), there are no identifiable differences in the number of GnRH-1-ir cell bodies in individuals maintained under long or short day lengths (Urbanski *et al.*, 1991). However, a rise in hypothalamic GnRH-1 content together with an increase in the size of GnRH-1 cell bodies has been detected in seasonally suppressed Syrian hamsters (Urbanski *et al.*, 1991). Together, these results suggest that breeding status may be linked to the concentration of hypothalamic GnRH-1 rather than the quantity of GnRH-1-ir cell bodies. Further investigation into the GnRH-1 system of naked mole-rats will help us elucidate the mechanisms of reproductive suppression in these animals.

The distribution of kisspeptin-ir cell bodies

Populations of kisspeptin-ir cell bodies are found at four distinct sites in the brains of naked mole-rats: the RP3V (consisting of the AVPV and PeN), PVH, the Arc and the DMH. In the female mouse, kisspeptin-ir cells are exclusively observed in the RP3V, the Arc and within the DMH (Clarkson and Herbison, 2006; Clarkson *et al.*, 2009b). Kisspeptin-ir cell bodies are found in the AVPV (only after colchicine injection) and Arc of rats (Adachi *et al.*, 2007). Interestingly, the distribution of kisspeptin-ir cell bodies in the brains of naked mole-rats is remarkably similar to that of laboratory mice. Like in mice, kisspeptin-ir cell bodies are distributed in the RP3V and PVH (Clarkson and Herbison, 2006; Clarkson *et al.*, 2009b).

Although kisspeptin-ir cell bodies are located in the RP3V continuum of both mice and naked mole-rats, there are some notable differences in the exact distribution of the cells. In mice, kisspeptin-ir cell bodies are present lining the wall of the 3V of mice in a tight band, whereas in naked mole-rats, most of the AVPV kisspeptin-ir cell

bodies extend laterally away from the wall of the 3V forming a more diffuse shape (Clarkson *et al.*, 2009b). In the PVH of naked mole-rats, kisspeptin-ir cell bodies are spread across its breadth. As in the case of mice and rats, the PVH is the only site for oxytocin-ir cell bodies in the naked mole-rat brain (Kalamatianos *et al.*, 2010). Since oxytocin is implicated in diverse social behaviours in other rodents (Veenema and Neumann, 2008), including naked mole-rats (Kalamatianos *et al.*, 2010), it would be intriguing to determine whether other social species, such as prairie voles and marmoset monkeys also show kisspeptin-immunoreactivity in the PVH.

Kisspeptin-ir cell bodies are also present in the Arc of naked mole-rats, surrounded by a dense network of kisspeptin-ir processes. This thick plexus has been reported in mice where it also obstructs the visualisation of kisspeptin-ir cell bodies in the Arc (Clarkson and Herbison, 2006; Clarkson *et al.*, 2009b). More controversially, this study indicates that there are kisspeptin-ir cell bodies in the DMH of naked mole-rats. The existence of DMH kisspeptin-ir cell bodies has also been confirmed in mice (Clarkson and Herbison, 2006; Clarkson *et al.*, 2009b). Despite this, the authenticity of these cell bodies must be questioned since other RFamide peptides are also present in the DMH and the use of nonspecific kisspeptin antibodies has previously resulted in the reporting of false-positive kisspeptin-ir cell bodies in the DMH (Brailoiu *et al.*, 2005). Moreover, *Kiss1* mRNA-expressing cells have not been reported in the DMH of mice (Gottsch *et al.*, 2004; Smith *et al.*, 2005a). Studies on mice have reported a scattered population of kisspeptin-ir cell bodies in the DMH of wild-type mice, but not in *Kiss1* KO mice (Clarkson *et al.*, 2009b). Furthermore, pre-adsorption of the kisspeptin antibody with RFRP-3 peptide does not neutralise the kisspeptin immunoreactivity in the DMH of mice (Clarkson *et al.*, 2009b).

In this present study, I report a scattered population of kisspeptin-ir cell bodies in the DMH of naked mole-rats. Pre-adsorption of the kisspeptin antibody with kisspeptin-10 peptide completely neutralises kisspeptin immunoreactivity in the DMH of naked mole-rats. However, pre-adsorption of the kisspeptin antibody with either the RFRP-1 or RPRF-3 peptide fails to neutralise the immunoreactivity. Although the present study indicates specificity for kisspeptin-immunoreactivity in the DMH of naked mole-rats, the functional significance of this cell population in any species remains obscure.

The distribution of kisspeptin-ir processes

In the rostral naked mole-rat brain, kisspeptin-ir processes are located in the MS, the HDB, the VDB, the NAcc and the VLS. In the MS and RP3V region of naked mole-rats, kisspeptin-ir processes form long bundles of parallel fibres streaming dorsoventrally close to the midline. More ventrally, kisspeptin-ir processes are distributed laterally

along the base of the naked mole-rat hypothalamus in the vicinity of kisspeptin-ir cell bodies. In naked mole-rats, kisspeptin-ir processes are contained within the PVH, also there is a complete absence of kisspeptin-ir processes in the SCN and VMH. More caudally, kisspeptin-ir processes are distributed abundantly in the Arc and ME, with a sparse scattering of kisspeptin-ir processes in the DMH.

Interestingly, the distribution of kisspeptin-ir process in naked mole-rats is remarkably similar to that of mice and rats, though there are slight differences in the distribution (Clarkson *et al.*, 2009b; Desroziers *et al.*, 2010). In the rostral forebrain of naked mole-rats, lone bundles of kisspeptin-ir processes are located in distinct regions; the VDB and HDB, the LS and NAcc. However, kisspeptin-ir processes in the forebrains of mice and rats form a continuum of fibres that extend dorsally from the LS, extending into the MS, surrounding the anterior commissure (AC), and finally extending ventrally into the HDB and VDB (Clarkson *et al.*, 2009b; Desroziers *et al.*, 2010). Furthermore, in mice, kisspeptin-ir processes are found outside the hypothalamus in the BNST, MeA, subfornical organ, periaqueductal grey and around the locus coeruleus (Clarkson *et al.*, 2009b). In the external zone of the ME, kisspeptin-ir processes are present in naked mole-rats, absent from mice and absent from rats (Clarkson *et al.*, 2009b; Desroziers *et al.*, 2010).

In naked mole-rats, there are numerous kisspeptin-ir processes in both the MPOA/HDB/VDB and ME, where GnRH-1-ir cell bodies and processes are found, respectively. The precise distribution of kisspeptin-ir processes is of significance for elucidating the mechanisms by which kisspeptins regulate GnRH-1 release. Firstly, kisspeptin-ir processes may project synaptically to GnRH-1-ir cell bodies to stimulate GnRH-1 release into the portal blood stream (Gottsch *et al.*, 2006). Secondly, kisspeptin-ir processes may project directly onto GnRH-1 neurone terminals at the level of the external zone of the ME to potentiate GnRH-1 secretion (Franceschini *et al.*, 2006; d'Anglemont de Tassigny *et al.*, 2008). Thirdly, the external zone of the ME may be a primary site for hypophysiotropic activity of kisspeptin. Kisspeptin may be released directly into portal circulation to influence LH/FSH secretion (Franceschini *et al.*, 2006). Finally, the presence of kisspeptin-ir processes in numerous brain regions could indicate indirect modes of action of kisspeptin on GnRH activity and the function of these kisspeptin-ir regions remains to be investigated.

A common problem with kisspeptin-ir staining is the cross-reactivity with other RFamide peptides which are related to kisspeptin (Oakley *et al.*, 2009). Previous rat and mouse studies mentioned above took special care to eliminate nonspecificity of kisspeptin antibodies by using two different antibodies (Desroziers *et al.*, 2010) or by testing the antibody specificity on *Kiss1* KO mice (Clarkson *et al.*, 2009b). In this present study, the kisspeptin immunoreactivity (including kisspeptin-ir cell bodies in the

DMH) is neutralised when the kisspeptin antibody is pre-adsorbed with the kisspeptin-10 peptide. However, pre-adsorption of the kisspeptin antibody with either the RFRP-1 or RFRP-3 peptide fails to neutralise the kisspeptin-immunoreactivity (including kisspeptin-ir cell bodies in the DMH). This indicates that all the kisspeptin-immunoreactivity observed in the naked mole-rat brain is specific to kisspeptin and not to other nonspecific RFamides.

Number of kisspeptin-ir cell bodies

Naked mole-rats in all eight animal groups display a relatively high number of kisspeptin-ir cells in the RP3V, PVH, cArc and DMH. One-way ANOVA test reveals a significant effect of animal group on the number of kisspeptin-ir cell bodies in the RP3V. Overall, there is a significant difference in the number of kisspeptin-ir cell bodies in both the RP3V and PVH between breeding/reproductively-activated animals and subordinates. Therefore, there is a significant effect of reproductive status on the number of kisspeptin-ir cell bodies in the RP3V and PVH. Nevertheless, despite having a suppressed HPG axis, subordinate mole-rats still had moderate numbers of kisspeptin-ir cell bodies in the RP3V and PVH.

Previous studies have implicated the RP3V region in the reproductive functioning of kisspeptin in rodents (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Clarkson and Herbison, 2006; Kauffman *et al.*, 2007). In female mice and rats, RP3V *Kiss1* neurons are thought to induce the preovulatory GnRH/LH surge that activates the HPG axis and leads to ovulation (Smith *et al.*, 2006; Clarkson *et al.*, 2008). Indeed, all kisspeptin-immunoreactivity is absent from the brains of *Kiss1* KO mice (Clarkson *et al.*, 2009b), this is not surprising given that mice with mutations in their *Kiss1* gene fail to undergo puberty (d'Anglemont de Tassigny *et al.*, 2007; Lapatto *et al.*, 2007). Although subordinate naked mole-rats are in a similar state of hypogonadotrophic hypogonadism to *Kiss1* KO mice, their kisspeptin system is merely suppressed and not absent like in *Kiss1* KO mice. This suppression (but not absence) may indeed relate to the temporary physiological and behavioural reproductive suppression of subordinates when in close proximity to queen (Jarvis, 1991). Therefore, when released from the suppressing effects of the queen (i.e. reproductively-activated), the kisspeptin system in the RP3V and PVH becomes upregulated, potentially triggering puberty-onset.

Previous studies have shown that both male and female subordinate naked mole-rats exhibit reduced pituitary LH response to exogenous GnRH-1 injections (Faulkes *et al.*, 1990b; Faulkes *et al.*, 1991), indicating that reproductive suppression is at the level of the hypothalamus rather than the pituitary. Taken together with the results from this present study; naked mole-rats have a suppressed pituitary gonadotrophin response, normal number of GnRH-1-ir cell bodies, significantly lower

number of kisspeptin-ir cells in the RP3V and significantly lower number of kisspeptin-ir cells in the PVH. Despite the lack of significant difference in the number of GnRH-ir cell bodies between reproductively-activated/breeding and subordinate naked mole-rats, the results from this study indicates a significant positive correlation between the number of GnRH-1-ir cells bodies and the number of kisspeptin-ir cell bodies in the RP3V, and a similar significant positive correlation is observed with kisspeptin-ir cell bodies in the PVH. This suggests that perhaps there is some attenuation in the GnRH system of subordinate naked mole-rats. Indeed, both *Kiss1*/*Gpr54* KO mice contain normal numbers of GnRH-1-ir cells and brain GnRH-1 content despite suffering from hypogonadotrophic hypogonadism (Messager *et al.*, 2005; d'Anglemont de Tassigny *et al.*, 2007; Clarkson *et al.*, 2008).

Like naked mole-rats, subordinate marmosets are reproductively suppressed by a single dominant female (Abbott *et al.*, 1998). Subordinate female marmosets are anovulatory, have low plasma oestrogen and progesterone levels, suppressed pituitary LH secretion and a reduced pituitary LH response to GnRH-1; moreover, reproductive suppression can be rapidly reversed when they are housed away from the dominant female (Abbott *et al.*, 1981; Abbott *et al.*, 1988). Following a GnRH-1 replacement therapy of multiple infusions, there is a rapid increase in plasma LH levels in subordinate anovulatory marmosets maintained within their peer group (and in presence of the dominant breeding female), and ovulation can be induced in 2 weeks (Abbott *et al.*, 1997). However, these females quickly revert to their hypogonadotrophic anovulatory condition upon cessation of the GnRH-1 treatment (Abbott *et al.*, 1997). Other theories for the reproductive suppression of ovulation in female marmoset monkeys, such as, stress-related theories, have been largely dismissed. For example, in subordinate female marmosets, plasma prolactin and cortisol concentrations are not increased above those of dominant females and the body masses of dominant and subordinate females are the same (Abbott *et al.*, 1988). So like in eusocial mole-rats, social suppression of fertility in subordinate females marmosets is mediated by reduced pituitary responsiveness of LH to GnRH-1.

So, reproductive suppression of subordinate naked mole-rats most likely occurs at the level of the RP3V/PVH kisspeptin system (this may also involve some attenuation in the GnRH-1 system), inhibiting LH/FSH secretion from the pituitary gland which ultimately leads to suppression of the HPG axis. This suppression of the RP3V/PVH kisspeptin system is removed when subordinates become reproductively-activated. It is interesting to speculate that changing social status from reproductively-activated to subordinate, or vice versa, simply operates a physiological 'switch' in the hypothalamus, turning GnRH-1 secretion 'off or 'on' respectively. It would be very interesting to investigate whether the RP3V/PVH kisspeptin system is suppressed in

other cooperatively-breeding animals, such as marmoset monkeys. This would determine whether reproductive suppression in cooperatively breeding animals could be mediated by a reduction in the number of kisspeptin expressing cells and a subsequent attenuated GnRH-1 release.

In all four regions where kisspeptin-ir cell bodies are observed (RP3V, PVH, cArc and DMH), there are no significant effects of gonadectomy on the number of kisspeptin-ir cell bodies. In mice and rats, gonadal steroids increase *Kiss1* expression in the RP3V (positive feedback pathway), whereas in the Arc these steroids inhibit *Kiss1* expression (negative feedback pathway) (Smith *et al.*, 2005a; Smith *et al.*, 2005b; Kauffman *et al.*, 2007). Conversely, removal of gonadal sex steroids after gonadectomy causes a decrease in *Kiss1* expression in the RP3V and an increase in *Kiss1* expression in the Arc (Smith *et al.*, 2005a; Smith *et al.*, 2005b; Kauffman *et al.*, 2007). Interestingly, photoinhibited seasonally breeding animals are also hypogonadotrophic. Expression of *Kiss1* in the Arc of ovariectomised ewes increases significantly during the transition from the anoestrous season to the breeding season, (Smith *et al.*, 2007). In the Syrian hamster (*Mesocricetus auratus*) *Kiss1* is expressed in the AVPV and Arc of adult sexually active hamsters raised in a long day photoperiod, yet *Kiss1* expression is significantly decreased in the Arc of sexually inactive animals in short day lengths (Revel *et al.*, 2007). Castration of long day (sexually active) hamsters significantly increases the expression of *Kiss1* in the Arc (Revel *et al.*, 2007).

The reproductive phenotype of subordinate naked mole-rats resembles that of other hypogonadotrophic animals we have previously discussed, such as *Kiss1*/*Gpr54* KO mice, subordinate marmosets and photoinhibited seasonally breeding animals, all of whom have low circulating gonadotrophins, sex steroids, and small gonads. Intriguingly, like naked mole-rats, *Kiss1*/*Gpr54* KO mice, subordinate marmosets and photoinhibited hamsters all have normal GnRH expression, and GnRH administration is still able to trigger normal LH/FSH release (Abbott *et al.*, 1988; d'Anglemont de Tassigny *et al.*, 2007; Revel *et al.*, 2007; d'Anglemont de Tassigny *et al.*, 2008). In naked mole-rats, the maintenance of high numbers of kisspeptin-ir cell bodies in GDX breeders indicates that these cell populations are relatively independent from gonadal hormone control. The lack of significant effect of gonadectomy on the number of kisspeptin-ir cell bodies in all four brain regions of naked mole-rats represents a significant divergence from the kisspeptin regulatory model observed in the animals studied so far. Naked mole-rats may lack the positive and negative steroidal pathways regulating the kisspeptin system, rather, the kisspeptin system may be regulated in alternative ways.

There is a lack of significant sex differences in the number of kisspeptin-ir cell bodies in the RP3V, PVH and cArc. In line with our findings on the maintained high

numbers of kisspeptin-ir cell bodies, other studies have also discovered a lack of sex differences on the neural morphology of naked mole-rats (Holmes *et al.*, 2009). In rodents, RP3V *Kiss1* neurones sexually differentiated, whereby adult female rats and mice have significantly more *Kiss1* expression and kisspeptin-ir cell bodies in the RP3V than adult males (Clarkson and Herbison, 2006; Kauffman *et al.*, 2007). The lack of significant effect of breeding history, gonadectomy or sex in these highly social species may reflect a general reduced influence of gonadal hormones on some functions to which these hormones are usually tightly linked. The ability of naked mole-rats to reproduce may be determined by their social status rather than by gonadal hormones and it is possible that in these highly social animals, social cues can be substitutes for some 'traditional' roles of gonadal hormones. It would be interesting to see whether the kisspeptin system of other social animals are also relatively independent from gonadal hormones. Whilst the key neuroendocrine factors underlying fertility (such as, kisspeptin, GnRH, oestrogen and testosterone etc.) have been well documented in a diverse range of species, little is known about the 'rules' of fertility-onset in mammals with alternative reproductive strategies.

Steroidal hormone levels

Previous studies on NMRs have measured urinary concentrations of testosterone and progesterone in, respectively, males and females, and plasma concentrations of progesterone in females (Faulkes *et al.*, 1990a; Faulkes and Abbott, 1991; Faulkes *et al.*, 1991; Clarke and Faulkes, 1998). This is the first study to provide data on plasma levels of gonadal steroids in both sexes.

As expected, there is a clear sexual differentiation in plasma testosterone levels. Breeding/reproductively-activated male naked mole-rats have higher plasma testosterone than all other female groups, including female breeders. Also, breeding/reproductively-activated males have significantly higher levels of plasma testosterone than GDX breeding males. Subordinate male naked mole-rats have slightly lower, but not significantly lower, plasma testosterone levels than reproductively-activated naked mole-rats. This is not surprising given minimal effect of gonadectomy on the kisspeptin/GnRH system.

Plasma oestradiol levels were generally low in all animals tested, and there was no significant effect of group. Unexpectedly, gonadectomy did not reduce plasma oestradiol levels in females, in fact, the GDX female breeders had the highest plasma oestradiol concentration. This anomaly may be explained by the relatively high oestradiol levels found in the adrenals of GDX female breeders. Therefore, future studies determining plasma oestradiol concentrations should be done on

adrenalectomised female naked mole-rats to obtain an accurate measurement of circulating gonadal steroid levels.

As expected, breeding female naked mole-rats have significantly higher plasma progesterone levels than breeding males, GDX female breeders and GDX male breeders (Faulkes *et al.*, 1990a; Faulkes *et al.*, 1990b). Surprisingly, we find no significant differences in plasma progesterone or oestradiol levels between subordinate females and breeding/reproductively-activated females. The reproductive suppression of subordinate female naked mole-rats is profound; they are anovulatory and have undetectable levels of urinary and plasma progesterone, and when removed from the queen and paired with a male, subordinate females show an increase in urinary progesterone to a level commonly observed in breeding females (Faulkes *et al.*, 1990a; Faulkes *et al.*, 1990b; Jarvis, 1991). A reason for this unexpected result may be due to our sampling method across the reproductive cycle, which is estimated to last approximately 34 days (Faulkes *et al.*, 1990a). In other spontaneous ovulators, oestradiol levels peak prior to ovulation and are considerably lower throughout the rest of the cycle (Bakker and Baum, 2000). Since our breeding/reproductively-activated females were sampled randomly with respect to the phase of the reproductive cycle, it is possible that the window of elevated oestradiol was missed.

CHAPTER 4:

Analysis of hypothalamic RFamide neuronal systems in breeding and subordinate eusocial Damaraland mole-rats (*Fukomys damarensis*)

ABSTRACT

Damaraland mole-rats live in colonies of up to 41 individuals and exhibit an extreme form of socially-induced infertility among mammals. Within the colony, only one dominant female (the queen) and male (occasionally two) are capable of breeding, however, subordinate Damaraland mole-rats can become reproductively-activated if removed from their natal colonies and paired with individuals of the opposite sex. Kisspeptin and RFamide-related peptide-3 (RFRP-3) are two RFamide neuropeptides that appear to be critical in the regulation of the reproductive neuroendocrine axis. This study investigated whether there are changes in the numbers of RFamide-immunoreactive (ir) cell bodies when adult subordinates are released from socially-induced reproductive suppression. These neurones were assessed in six groups of animals: breeders, gonadectomised (GDX) breeders and subordinates, of both sexes. RFamide-ir cell bodies were located in the medial preoptic area (MPOA). Breeding and GDX animals had significantly more RFamide-ir cell bodies in the MPOA, in fact, nearly all subordinates had no RFamide-ir cell bodies in the MPOA. Furthermore, there was no effect of gonadectomy or sex on the number of RFamide-ir cell bodies. These results indicate that the transition of Damaraland mole-rats from subordinate to breeding status involves (1) upregulation of the MPOA RFamide system and (2) is relatively independent from gonadal hormones− this independence may also be a hallmark of eusociality. However, the identity of this RFamide peptide is not known since pre-adsorption tests with kisspeptin-10 peptide failed to neutralise the immunoreactivity. This study highlights a novel non-specificity problem with a kisspeptin antibody (from A. Caraty) widely used in reproduction research. The identity of this RFamide peptide detected is most likely another member of the RFamide family of peptides.

INTRODUCTION

Eusociality is a highly successful social strategy well-known amongst social insects, with only two mammalian species known to be eusocial (Lacey and Sherman, 1991). Within the colony of Damaraland mole-rats, only one dominant female (the queen) and male (occasionally two) are capable of breeding, and the vast majority of individuals will remain subordinate (Bennett, 1990). As in the naked mole-rats, the reproductive hierarchy of Damaraland mole-rats is rigid, but subordinate Damaraland mole-rats are not sterile and can become reproductively-activated if removed from their natal colony and paired with individuals of the opposite sex (Bennett and Jarvis, 1988). This is our routine procedure for creating reproductively-activated animals in the laboratory.

(Refer to chapter 1 for general introduction on reproductive suppression in Damaraland mole-rats).

Kisspeptin and RFamide-related peptide-3 (RFRP-3) are two RFamide neuropeptides that appear to be critical in the regulation of the reproductive neuroendocrine axis (Smith *et al.*, 2008). The HPG axis controls fertility via a coordinated balance of stimulatory and inhibitory neuroendocrine systems− ultimately driving GnRH secretion. In 2000, a novel avian hypothalamic peptide capable of inhibiting gonadotrophin secretion in cultured quail pituitary cells was discovered, and this hormone was named gonadotrophin-inhibitory hormone (GnIH) (Tsutsui *et al.*, 2000). Soon after, the presence and function of the ortholog of this GnIH, RFRP-3 was confirmed in mammals. In avian species, GnIH injections resulted in a decrease in LH release and rapid suppression of female sexual behaviour (Osugi *et al.*, 2004; Bentley *et al.*, 2006). Moreover, GnIH fibres formed close contacts with GnRH-1 neurones in the POA in birds, suggesting that the modulation of the reproductive axis occurs at the level of the brain (Bentley *et al.*, 2003; Ubuka *et al.*, 2008). GnIH cells are found only in the PVH with extensive fibres projecting caudally to the ME to directly regulate pituitary gonadotrophin secretion (Bentley *et al.*, 2003; Ukena *et al.*, 2003; Ubuka *et al.*, 2008).

In rats, mice and Syrian hamsters, RFRP-3-ir cell bodies and cells expressing RFRP-3 were distributed in the DMH, with no other brain regions showing evidence of cell body staining (Kriegsfeld *et al.*, 2006). Furthermore, about 40% of GnRH cells received projections from RFRP-3-ir fibres, indicating the potential for direct inhibitory control. To date, RFRP-3 has been identified in the brains of all mammalian species investigated. In mice, direct application of RFRP-3 to GnRH cells in cultured brain slices decreases firing rate in a sub-population of cells (Ducret *et al.*, 2009). In ewes, peripheral administration of the RFRP-3 reduces ovine LH pulse amplitude and

suppresses LH and FSH release *in vitro* (Clarke *et al.*, 2008). Thus, RFRP-3 plays a powerful role in controlling the HPG axis in animals.

In chapter 3, I analysed GnRH-1- and kisspeptin-immunoreactivity in the eusocial naked mole-rat brain. In this present study, I compared RFamide-immunoreactivity in breeding and subordinate Damaraland mole-rats (the other eusocial mole-rat) to elucidate their potential roles in HPG axis activation. Furthermore, the effects of reproductive status, gonadectomy and sex on the number of immunoreactive cell bodies of an uknown RFamide were tested. To confirm the specificity of the kisspeptin antibody, a series of pre-adsorption tests were done to determine any potential cross-reactivity with other RFamides.

MATERIALS AND METHODS

Study animals

The current experiment compared six groups of Damaraland mole-rats: (group 1) female breeders (N= 7); (group 2) male breeders (N= 7); (group 3) female GDX breeders (N= 4); (group 4) male GDX breeders (N= 3); (group 5) female subordinates (N= 5) and (group 6) male subordinates (N= 6). Male breeders were significantly heavier than females breeders and subordinates of both sexes ($p < 0.05$).

Kisspeptin immunohistochemistry

One series of sections was immunostained for kisspeptin (polyclonal rabbit anti-kisspeptin 1:15,000; A. Caraty no. 564) as described in chapter 3 for GnRH-1 immunohistochemistry (minus the treatment with 0.1% sodium borohydride). The secondary antibody used was biotinylated donkey anti-rabbit IgG (1:1000; Stratech, Newmarket, Suffolk, UK).

Controls and specificity

Control procedure included omission of the primary kisspeptin antibody, pre-adsorbing the kisspeptin antibody overnight with mouse kisspeptin-10 peptide 1 µg/ml (YNWNSFGLRY-NH2; Phoenix Pharmaceuticals, Peterborough, UK). To determine whether the kisspeptin antibody cross-reacts with other RFamide peptides, it was incubated with RFRP-1 peptide 10 µg/ml (VPHSAANLPLRF-NH2, Phoenix Pharmaceuticals) or RFRP-3 peptide 10 µg/ml (VPNLPQRF- NH2, Phoenix Pharmaceuticals).

Digital photomicrographs

Brightfield photomicrographs were obtained using a Nikon E600 microscope at magnifications of x40, x100 and x200 with a Micro-Publisher 5.0 camera (InterFocus Imaging, Cambridge, UK). The camera was controlled by MCID Core software (Interfocus Imaging). Images were later post-processed using Adobe Photoshop, CS3 to adjust brightness and contrast. Background artefacts were removed as necessary; no other modifications were made to images. Final images were compiled into multi-panel plates in Microsoft Publisher, minor changes to brightness and contrast were made after importing into Microsoft Publisher if necessary.

Quantification and statistical analysis

Slides were examined and counted under bright field illumination using a microscope using a Nikon E600 microscope at X200 magnification. For each animal, the mean

number of RFamide-ir cell bodies in the MPOA was established using anatomically matched brain sections. I did not count the number of positively stained cells in the Arc since the cells were not discernable against the high degree of background staining (See Plate 4.1A2 inset).

For all comparisons, statistical significance was set at $p< 0.05$. Kolgomorov-Smirnoff test determined that our data was non-parametric; $Z= 1.421$, $p= 0.035$. For each dependent measure, an overall Kruskal-Wallis test was performed on all six animal groups. Significant differences between the six groups were assessed by Mann-Whitney U tests. A Pearson's correlation between body mass and number of MPOA RFamide-ir cell bodies was undertaken.

RESULTS

Tests for specificity of the immunoreactivity

Pre-adsorption of the kisspeptin antibody (A. Caraty no. 564) with the kisspeptin-10 failed to neutralise the immunoreactivity in the MPOA (Plate 4.1B1). In the Arc, pre-adsorption of the kisspeptin antibody with kisspeptin-10 resulted in neutralisation of the immunoreactivity (Plate 4.1B2). Pre-adsorption of the kisspeptin antibody with RFRP-1 did not result in any attenuation of the signal in the MPOA (Plate 4.1C1) or Arc (Plate 4.1C2). Pre-adsorption of the kisspeptin antibody with RFRP-3 also did not result in any attenuation of the signal in the MPOA (Plate 4.1D1) or Arc (Plate 4.1D2). These results indicate that the kisspeptin antibody was not staining specifically for kisspeptin, RFRP-1 or RFRP-3 in the MPOA, rather, kisspeptin antibody was staining for another unknown RFamide. In the Arc, pre-adsorption of the kisspeptin antibody with kisspeptin-10, but not with the RPRP-1 or RFRP-3 peptide, resulted in the absence of immunoreactivity. Therefore, these results suggest that the immunoreactivity in the Arc is kisspeptin, however, the stained cells in the MPOA are an unknown RFamide peptide.

Distribution of kisspeptin-ir cell bodies

Kisspeptin-ir cell bodies were located throughout the Arc surrounded by scattered kisspeptin-ir processes (Plate 4.2A2). Kisspeptin-ir cell bodies were difficult to discern due to the density of kisspeptin-ir processes in the Arc.

Distribution of kisspeptin-ir processes

Kisspeptin-ir processes were scattered at high density throughout the Arc, extending ventrally into the ME (Plate 4.2B1-B2). In the ME, kisspeptin-ir processes formed an extensive arching pattern around the ME that continues into the PS. No other region displayed kisspeptin-ir processes. There were no differences in the distribution of kisspeptin-ir processes between any of the groups.

Distribution of RFamide-ir cell bodies

RFamide-ir cell bodies were loosely distributed along the septo-preoptico-hypothalamo pathway (Plate 4.2A1-A2). The highest density of RFamide-ir cell bodies were located in the MPOA, dorsally, a small scatter of these cells were located in the MS. Within the MPOA and MS, RFamide-ir cells were distributed diffusely, with cells located just laterally to these regions. There were no aggregations of RFamide-ir cell bodies around the base of the hypothalamus or the wall of the 3V.

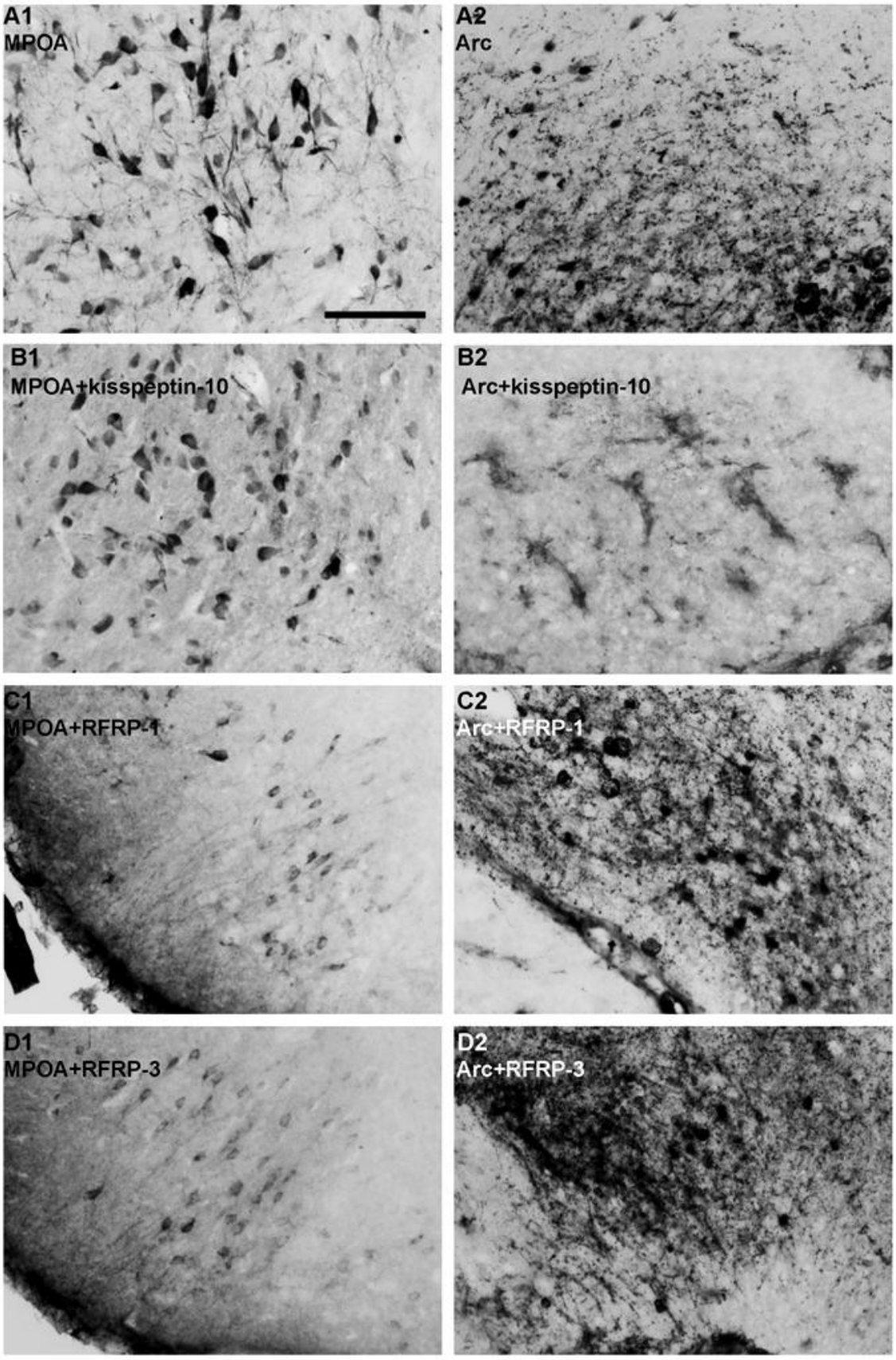


Plate 4.1: Brightfield photomicrographs of representative coronal sections showing staining with the kisspeptin antibody (A1) in the MPOA, (A2) in the Arc, (B1) pre-adsorbed with kisspeptin-10 peptide in the MPOA, (B2) pre-adsorbed with kisspeptin-10 peptide in the Arc, (C1) pre-adsorbed with RFRP-1 peptide in the MPOA, (C2) pre-adsorbed with RFRP-1 peptide in the

Arc, (D1) pre-adsorbed with RFRP-3 peptide in the MPOA and (D2) pre-adsorbed with RFRP-3 peptide in the Arc of a breeding female Damaraland mole-rat. Males are not shown but their distribution is the same. Arc, arcuate nucleus; MPOA, medial preoptic area. Scale bars= 100µm in A1-D2.

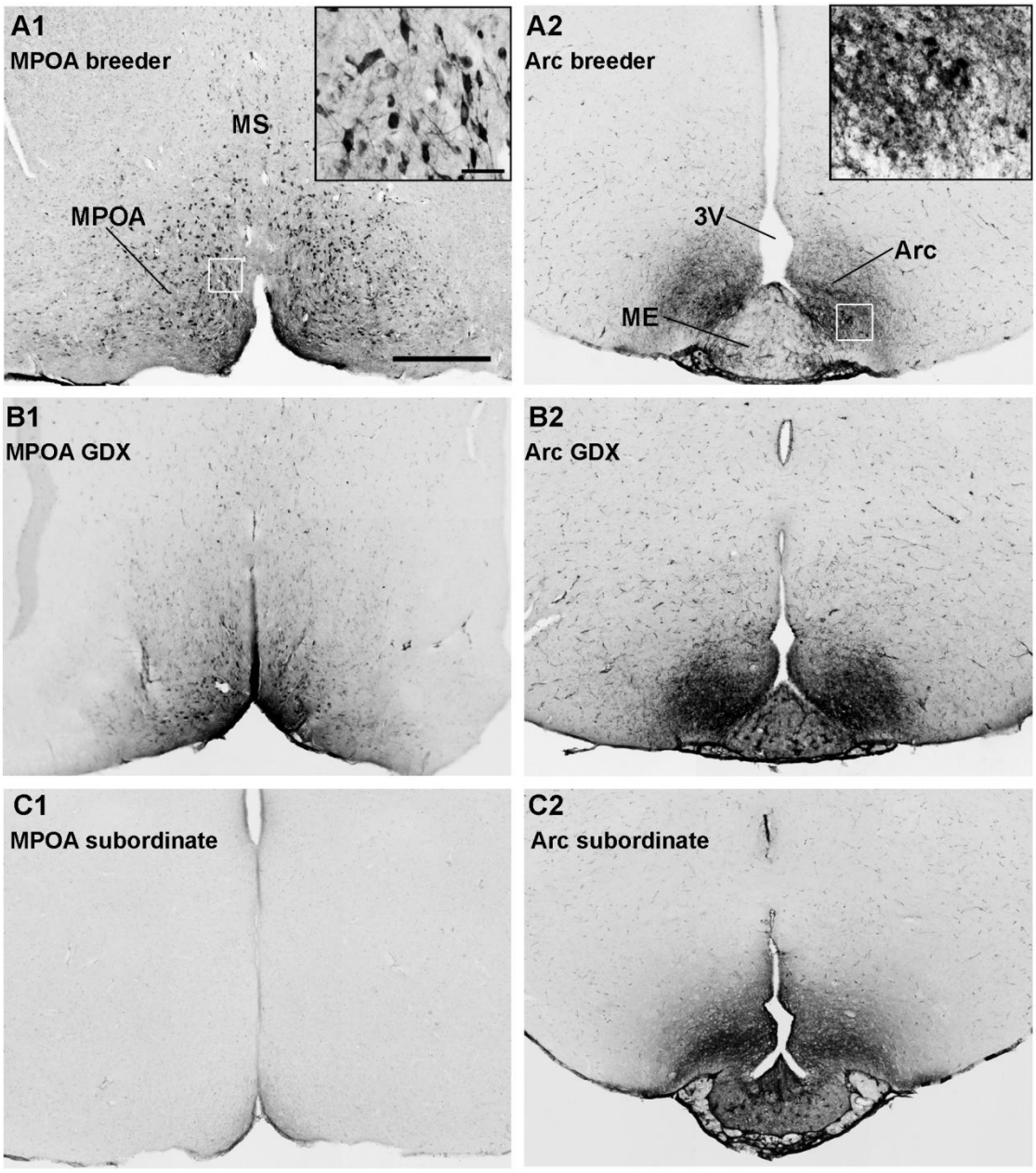


Plate 4.2: Brightfield photomicrographs of representative coronal sections of Damaraland mole-rats showing RFamide-immunoreactivity in (A1) the MPOA of a breeding female, (A2) the Arc of a breeding female, (B1) the MPOA of a GDX breeding female, (B2) the Arc of a GDX breeding female, (C1) the MPOA of a subordinate female and (C2) the Arc of a subordinate female. Males are not shown but their distribution is the same. Boxed regions are shown in higher magnification insets in the same photomicrograph. Scale bars= 500µm in A1-C2; 50µm in A1 and A2 inset. 3V, third ventricle; Arc, arcuate nucleus, GDX, gonadectomised, ME, median eminence, MPOA, medial preoptic area; MS, medial septum.

Number of RFamide-ir cell bodies in the MPOA

Within breeding animals, there was a large variation in the mean number of RFamide-ir cell bodies in the MPOA− ranging from 0 to 923. Of the 7 female breeding animals (group 1); one animal contained no MPOA RFamide-ir cells, another animal contained 92 (relatively low) MPOA RFamide-ir cells and the remaining animals contained between 157-366 (relatively high) MPOA RFamide-ir cells. Of the 7 male breeding animals (animal group 2), two animals contained no MPOA RFamide-ir cells, another contained 99 (relatively low) MPOA RFamide-ir cells and the remaining animals contained between 217-435 (relatively high) MPOA RFamide-ir cells. There was a large variation in the number of MPOA RFamide-ir cells for both the gonadectomised groups, ranging from 3 to 923. Almost all of the subordinate animals displayed an absence of RFamide-ir cell bodies in the MPOA. Therefore, these observations suggest that about half of the breeders had high amounts of RFamide-ir cell bodies and the other half had a low numbers or an absence of RFamide-ir cell bodies. In contrast, virtually all subordinate animals contained no RFamide-ir cell bodies, one subordinate animal had 5 RFamide-ir cells and all the other subordinates contained no RFamide-ir cells (Figure 4.1).

A Kruskal-Wallis test revealed that there was a significant effect of animal group on the number RFamide-ir cell bodies in the MPOA; $H(5)= 16.288$, $p= 0.006$. There was no significant effect of gonadectomy or sex on the number of RFamide-ir cell bodies in the MPOA. A series of Mann-Whitney U tests revealed a significant effect of reproductive status on the number of RFamide-ir cell bodies in the MPOA. Subordinate females (group 5) had significantly lower number of RFamide-ir cell bodies in the MPOA than: female breeders (group 1; $U= 3.000$, $Z= -2.442$, $p= 0.015$), male breeders (group 2; $U= 6.000$, $Z= -1.994$, $p= 0.046$), female GDX breeders (group 3; $U= 1.000$, $Z= -2.303$, $p= 0.021$) and male GDX breeders (group 4; $U= 0.000$, $Z= -2.382$, $p=0.017$) (Figure 4.1). Subordinate males (group 6) had significantly lower number of RFamide-ir cell bodies in the MPOA than: female breeders (group 1; $U= 3.000$, $Z= -2.795$, $p= 0.005$), male breeders (group 2; $U= 6.000$, $Z= -2.443$, $p= 0.015$), female GDX breeders (group 3; $U= 0.000$, $Z= -2.882$, $p= 0.004$) and males GDX breeders (group 4; $U= 0.000$, $Z= -2.761$, $p= 0.006$) (Figure 4.1). Furthermore, there was a significant positive correlation between body mass and the number RFamide-ir cells (Figure 4.2; $r= 0.458$, $p= 0.021$). This is not surprising given that male breeders are significantly heavier than female breeders and subordinate of both sexes.

The limitations in the quantification of RFamide-immunoreactivity in Damaraland mole-rats are discussed below.

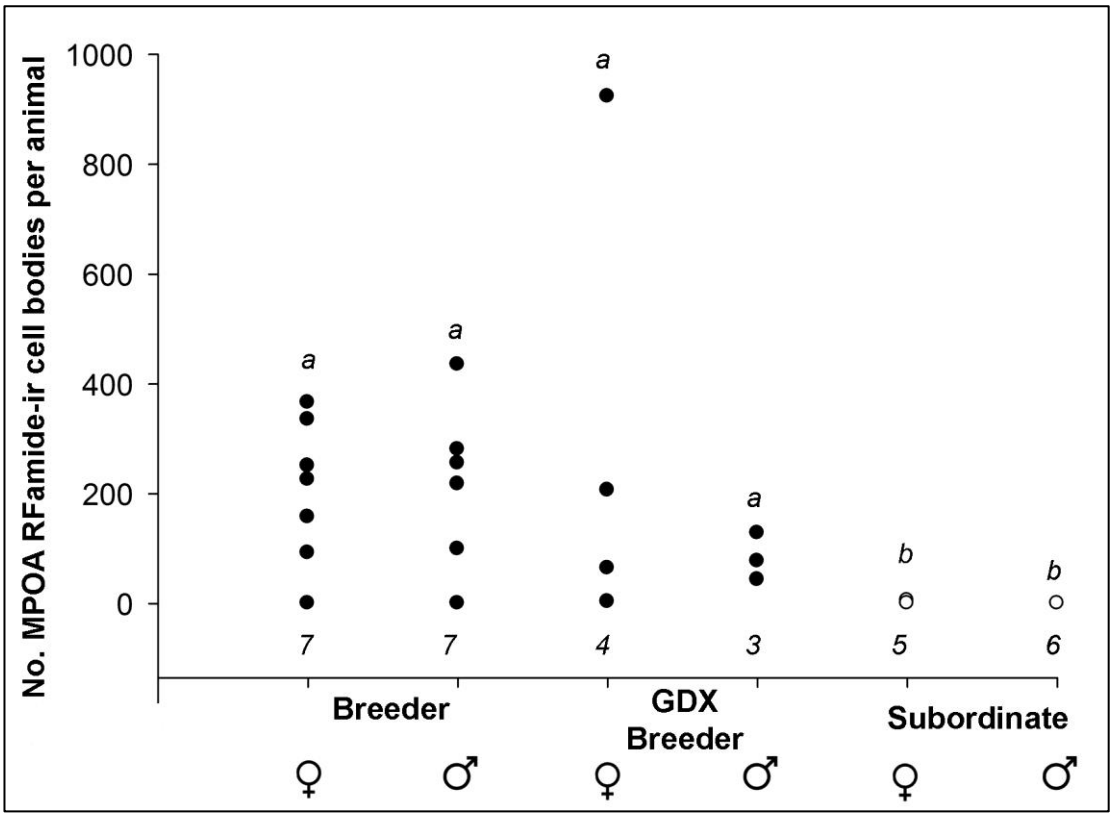


Figure 4.1: The number of MPOA RFamide-ir cells in breeders (females and males), gonadectomised (GDX) breeders (females and males) and subordinates (females and males). Number of animals per group is noted at the base in italics. ♀= female animal group; ♂= male animal group. Black filled circles represent breeders; unfilled circles represent subordinates. *a* and *b* indicates statistical significance at the $p < 0.05$ level.

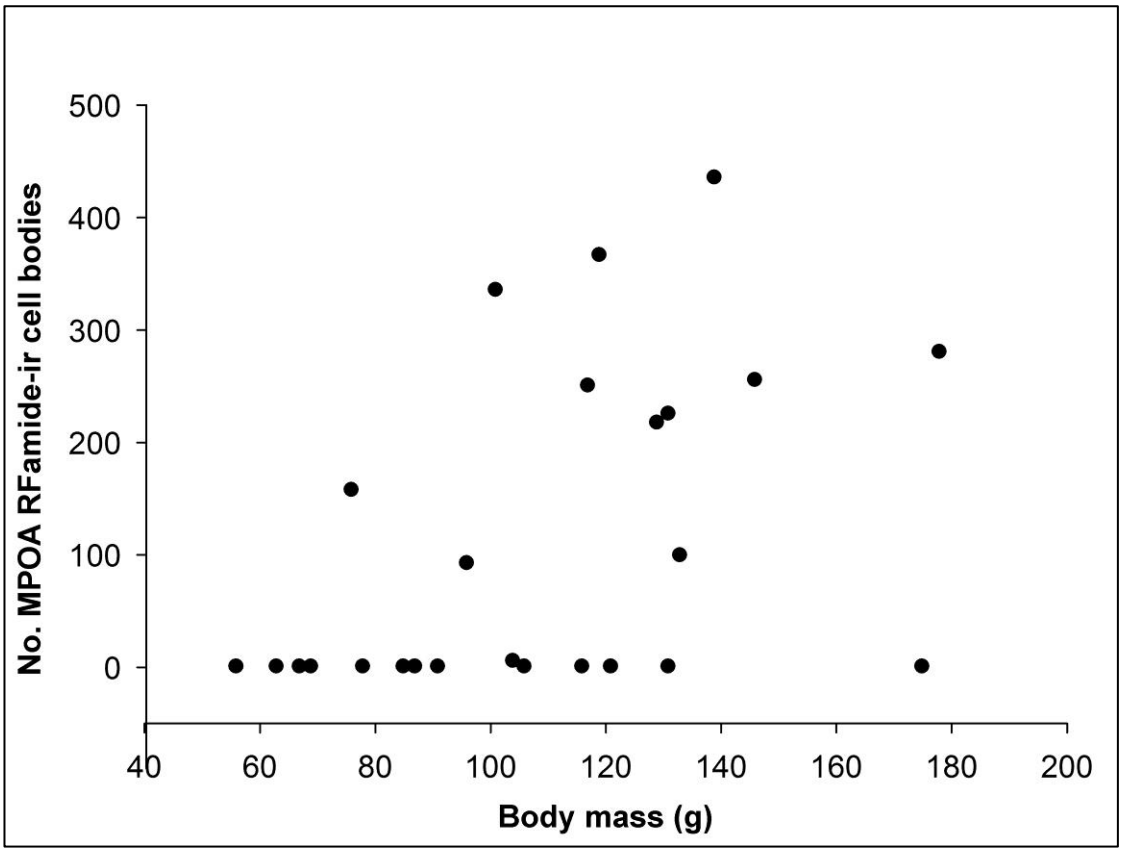


Figure 4.2: Significant positive correlation between body mass (g) and number of MPOA RFamide-ir cell bodies per animal

DISCUSSION

Damaraland mole-rats offer a unique model through which the effects of social environment on HPG axis-activation can be studied in the laboratory. Damaraland mole-rats are an extreme example of not only cooperative breeding, but also of socially-induced infertility (Bennett and Jarvis, 1988; Bennett, 1990). The physiological systems required for reproduction in female subordinates are suppressed by the presence of the queen (Bennett and Jarvis, 1988; Bennett, 1994). Kisspeptin influences the reproductive system through the HPG axis where it assists the release of GnRH from the hypothalamus, and ultimately the release of LH and FSH from the pituitary gland (Gottsch *et al.*, 2004).The present study investigated the possibility that immunohistochemically identifiable changes in the neuronal systems containing kisspeptin contribute to the onset of fertility when subordinate Damaraland mole-rats are removed from the reproductive suppressing effects of the queen.

Specificity of the immunoreactivity

Since kisspeptin is a member of a large family of RFamide peptides, there has been a lot of controversy surrounding the specificity in the immunohistochemical method of detecting kisspeptin neurones. In 2005, a study mapped the complete distribution of kisspeptins in the brains of rats of both sexes (Brailoiu *et al.*, 2005). The rabbit polyclonal antibody used in the former study (from Phoenix Pharmaceuticals Inc., Belmont, CA, USA) was later shown to cross-react with a number of other RFamide peptides (Goodman *et al.*, 2007). Pre-adsorption of this kisspeptin antibody (from Phoenix Pharmaceuticals) with kisspeptin-10 peptide neutralised the staining, however, pre-adsorption of this same antibody with other RFamides also completely blocked the immunostaining (Goodman *et al.*, 2007). These results concluded that the kisspeptin antibody (from Phoenix Pharmaceuticals) was non-specific and cross-reacted with other peptides in the RFamide family.

To eliminate the problem of cross-reactivity, a more specific kisspeptin antibody raised in rabbits against mouse kisspeptin-10 (from A. Caraty) has been used in numerous kisspeptin-immunoreactivity studies (Franceschini *et al.*, 2006; Clarkson *et al.*, 2009b; Desroziers *et al.*, 2010). An extensive series of pre-adsorption experiments have confirmed that this kisspeptin antibody (from A. Caraty) only specifically stains for kisspeptin, and not other members of the RFamide family of peptides, such as RFRP-3, GnIH, NFF, Chemerin, QRFP and PrRP (Franceschini *et al.*, 2006; Goodman *et al.*, 2007; Clarkson *et al.*, 2009b). When this antibody (from A. Caraty) was pre-adsorbed with RFRP-3 peptide at a concentration that has been shown to completely neutralise RFRP-3-immunoreactivity, there was no change to kisspeptin-immunoreactivity

throughout the mouse brain (Clarkson *et al.*, 2009b). Also, when this kisspeptin antibody (from A. Caraty) was used on *Kiss1* KO mice, no kisspeptin-immunoreactivity was detected anywhere in the brain (Clarkson *et al.*, 2009b). In chapter 3 of this thesis, kisspeptin-immunoreactivity was completely neutralised in the naked mole-rat brain when the kisspeptin antibody (from A. Caraty) was pre-adsorbed with the kisspeptin-10 peptide. Also, pre-adsorption of the kisspeptin antibody with either the RFRP-1 or RFRP-3 peptide failed to neutralise the kisspeptin-immunoreactivity in the naked mole-rat brain. Together, these neutralisation and *Kiss1* KO mice experiments indicate that the rabbit polyclonal kisspeptin antibody (from A. Caraty) specifically identifies kisspeptin and not other RFamides.

In this present study, pre-adsorption of the kisspeptin antibody (A. Caraty no. 564) with the kisspeptin-10 peptide neutralised the immunoreactivity in the Arc of Damaraland mole-rats, however, there was no attenuation of the immunoreactivity in the MPOA of Damaraland mole-rats. This creates several problems for interpretating the results in this chapter and, moreover, makes identification of the stained MPOA cells difficult. There are several reasons which may explain why kisspeptin-immunoreactivity in the MPOA failed to become neutralised after being blocked with kisspeptin-10 peptide.

Firstly, the failure to neutralise MPOA immunoreactivity may be due to problems with the synthetic kisspeptin-10 peptide and/or blocking protocol. In chapter 3 of this thesis, the same batch of kisspeptin-10 peptide was used to successfully neutralise kisspeptin-immunoreactivity in naked mole-rats. Furthermore, the same batch of kisspeptin-10 peptide was also used to neutralise kisspeptin-immunoreactivity in the Arc of Damaraland mole-rats in this chapter, indicating that the failure to neutralise does not lie with the kisspeptin-10 peptide. These results indicate that failure to neutralise the immunoreactivity in the MPOA of Damaraland mole-rats cannot be explained by problems with the kisspeptin-10 peptide or the blocking protocol, such as, concentration of kisspeptin-10 peptide used.

Another reason for the failure of kisspeptin-10 peptide to neutralise immunoreactivity may be due to non-specificity of the kisspeptin antibody. The kisspeptin antibody (from A. Caraty) was raised against the mouse kisspepin-10 sequence YNWNSFGLRY-NH2. This same kisspeptin-10 amino acid sequence is also shared with the frog, opossum, rat, sheep, cattle and pig, whilst notable deviations from this mouse kisspeptin-10 amino acid sequence include humans and monkeys (Decourt *et al.*, 2008; Oakley *et al.*, 2009). Previous pre-adsorption studies on mice (Clarkson *et al.*, 2009b), rats (Desroziers *et al.*, 2010) and naked mole-rats (chapter 3 of this thesis) have resulted in the successful neutralisation of this antibody. These results indicate that this kisspeptin antibody specifically binds to the common mouse kisspeptin-10.

Therefore, the failure to neutralise immunoreactivity in the MPOA of Damaraland mole-rats makes it impossible to accurately identify the immunoreactive cell population in the MPOA. Although the identity of these MPOA cells remain inconclusive, it is most likely that these cells belong to the RFamide family of peptides. Henceforth, these immunoreactive cells in the MPOA of Damaralands mole-rats will be referred to as RFamide-ir cell bodies. In the future, it is hoped that the sequencing of the Damaraland mole-rat kisspeptin gene will provide new resources deducing the identity of these immunoreactivie MPOA cell bodies.

Distribution of RFamide-ir and kisspeptin-ir cell bodies

The results from this study indicate that kisspeptin-ir cell bodies are present in the Arc of Damaraland mole-rats. In rats, mice and Syrian hamsters, RFRP-3-ir cell bodies and cells expressing RFRP-3 were distributed in the DMH, with no other brain regions showing evidence of cell body staining (Kriegsfeld *et al.*, 2006). Furthermore, about 40% of GnRH cells received projections from RFRP-3-ir fibres, indicating the potential for direct inhibitory control. To date, RFRP-3 has been identified in the brains of all mammalian species investigated.

RFamide-ir cells are largely located in the MPOA of Damaraland mole-rats, with cells extending into the lateral margins of the MPOA and dorsally into the MS. This distribution is very intriguing since no previous studies have reported the presence of kisspeptin-ir cells in this location in rodents (Oakley *et al.*, 2009). In mice, kisspeptin-ir cell bodies and *Kiss1* neurones are located in the RP3V (consisting of the AVPV and PeN) and Arc (Gottsch *et al.*, 2004; Clarkson and Herbison, 2006; Clarkson *et al.*, 2009b). Also, kisspeptin-ir cell bodies are found in the DMH of mice, although there is no *Kiss1* expression in this region (Gottsch *et al.*, 2004; Clarkson and Herbison, 2006; Clarkson *et al.*, 2009b). In rats *Kiss1* neurones are located in the RP3V and Arc, furthermore, kisspeptin-ir cells are found in the AVPV (after colchicine treatment) and Arc (Irwig *et al.*, 2004; Smith *et al.*, 2006; Adachi *et al.*, 2007; Kauffman *et al.*, 2007; Desroziers *et al.*, 2010). In hamsters, *Kiss1* neurones and kisspeptin-ir cells are located in the Arc of Syrian hamsters, however, kisspeptin-ir cell bodies are located in the AVPV and Arc of Siberian hamsters (Revel *et al.*, 2006; Greives *et al.*, 2007; Mason *et al.*, 2007). Interestingly, kisspeptin-ir cell bodies are also found in the POA in ewes, an area known to contain numerous GnRH-1-ir cell bodies in sheep (Caldani *et al.*, 1988; Franceschini *et al.*, 2006). In addition, around 40% of GnRH-ir cell bodies in the POA of ewes had contact with kisspeptin-ir processes (Smith *et al.*, 2008). However, the relationship between GnRH-1-ir cell bodies and RFamide-ir cell bodies in the MPOA of Damaraland mole-rats remains to be determined.

Distribution of kisspeptin-ir processes

Unlike other rodents studied, kisspeptin- ir processes were only observed in the Arc of Damaraland mole-rats. In the MPOA/MS/HDB/VDB region of mice and naked mole-rats, long bundles of parallel kisspeptin-ir processes stream dorsoventrally close to the midline, and more ventrally, kisspeptin-ir processes are distributed laterally along the base of the brain (Clarkson and Herbison, 2006; Clarkson *et al.*, 2009b). This distribution is very different to what is observed in Damaraland mole-rats, where kisspeptin- ir processes are absent in the MPOA. So although a high proportion of GnRH-1-ir cell bodies are located in the MPOA of Damaraland mole-rats (Molteno *et al.*, 2004), none of these GnRH-1-ir cell bodies are in close apposition with any kisspeptin-ir neurones due to the absence of kisspeptin-immunoreactivity in this region. Moreover, in other rodents, kisspeptin-ir processes are located in the RP3V and PVH regions surrounding the kisspeptin-ir cell bodies (Clarkson *et al.*, 2009b; Desroziers *et al.*, 2010). The fact that kisspeptin-ir processes are not located in the RP3V/PVH region in Damaraland mole-rats may be related to the absence of kisspeptin-ir cell bodies in these regions. In other rodents, such as naked mole-rats, mice and rats, kisspeptin-ir processes form an extremely dense plexus of fibres in the Arc (Clarkson *et al.*, 2009b; Desroziers *et al.*, 2010). The impact of these Arc kisspeptin-ir processes on the efficacy of the HPG axis in Damaraland mole-rats remains to be seen.

Number of RFamide-ir cell bodies in the MPOA

There is a large variation in the number of RFamide-ir cell bodies in the MPOA of breeding Damaraland mole-rats. In contrast, almost all of the subordinate animals uniformly displayed an absence of RFamide-ir cell bodies in the MPOA. Overall, about half of the breeders had high numbers of RFamide-ir cell bodies and the other half had a low numbers or an absence of RFamide-ir cell bodies. On the contrary, virtually all subordinate animals contained no RFamide-ir cell bodies. Statistical tests confirmed that both subordinate groups had significantly lower number of MPOA RFamide-ir cell bodies than any of the breeding animal groups. Although it is surprising that some breeding animals contain no or very little number of MPOA RFamide-ir cell bodies, nevertheless, the finding that all four breeding animal groups have significantly higher number of RFamide-ir cells than both of the subordinate animal groups is remarkable.

Previous studies have implicated the RP3V region in the reproductive functioning of kisspeptin in rodents (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Clarkson and Herbison, 2006; Kauffman *et al.*, 2007) In female mice and rats, RP3V *Kiss1* neurones are thought to induce the preovulatory GnRH/LH surge that activates the HPG axis and leads to ovulation (Smith *et al.*, 2006; Clarkson *et al.*, 2008). Indeed, mice with mutations in their *Kiss1/Gpr54* gene fail to undergo puberty and all kisspeptin

immunoreactivity is absent from the brains of *Kiss1* KO mice (d'Anglemont de Tassigny *et al.*, 2007; Lapatto *et al.*, 2007; Clarkson *et al.*, 2009b). Previously in chapter 3 of this thesis, I discovered that kisspeptin cell in the RP3V and PVH are up-regulated when subordinate naked mole-rats become reproductively-activated. In Damaraland mole-rats, the presence of RFamide-ir cell bodies in breeders and the absence of RFamide-ir cell bodies in the subordinates suggests that when subordinate Damaraland mole-rats are released from reproductive suppression, there is an up-regulation of RFamide-ir cell bodies in the MPOA.

Earlier studies have shown that only female subordinate Damaraland mole-rats have physiologically suppressed reproductive systems. Female subordinate Damaraland mole-rats exhibit reduced pituitary LH response to exogenous GnRH-1 injections (Bennett *et al.*, 1993; Bennett *et al.*, 1996) Furthermore, after ovariectomy, reproductively-activated females continued to show significantly greater GnRH-1 stimulated LH secretion than subordinate females (Molteno and Bennett, 2000). These observations suggest that the relative insensitivity of female subordinates to GnRH may be due to insufficient chronic stimulation by endogenous GnRH at the level of the hypothalamus, and the differential inhibition of LH response between reproductive and subordinate females occurs independently of gonadal steroids. In contrast to females, no differences in urinary testosterone, plasma LH or the LH response to a GnRH challenge are found between subordinate and breeding males (Bennett *et al.*, 1993; Bennett, 1994). Furthermore, whilst female subordinate Damaraland mole-rats have higher GnRH concentrations in the hypothalamus than female breeders, there are no differences in GnRH concentrations between subordinate and breeding males (Molteno *et al.*, 2004). These results are consistent with the hypothesis that GnRH release is inhibited in the subordinate females but not in the subordinate males of this species, indicating that reproductive inhibition in subordinate males may be strictly behavioural.

The results from this present study indicate that both the female and male subordinate Damaraland mole-rats displayed significantly lower numbers of RFamide-ir cells. Although the pre-adsorption tests were not able to confirm the identity of these cells, it is suprising that these RFamide-ir cells are absent in both subordinate male and female Damaraland mole-rats. These results are inconsistent with previous findings in the above paragraph which show that only the female subordinates have suppressed reproductive systems. Subordinate male Damaraland mole-rats are still able to have normal GnRH and gonadotrophin system, and functional gonads. This observation differs to that of other infertile rodents, such as, *Kiss1* KO mice, whereby these mutants display no kisspeptin-immunoreactivity, have deficiencies in their HPG axis and fail to undergo puberty (Clarkson *et al.*, 2008; Clarkson *et al.*, 2009b).

There are no significant differences between the intact breeders and the GDX breeding Damaraland mole-rats. In fact, one of the GDX female breeders had the highest number of MPOA RFamide-ir cell bodies. This result is perhaps not so surprising given that GnRH-stimulated LH secretion has previously been shown to be independent from gonadal steroids (Molteno and Bennett, 2000). Previous studies have shown that the kisspeptin system of rats, mice, rodents and sheep are all modulated by gonadal steroids, as indicated by gonadectomy experiments (Smith *et al.*, 2005a; Smith *et al.*, 2005b; Kauffman *et al.*, 2007; Revel *et al.*, 2007; Smith *et al.*, 2007). Interestingly, I have previously shown that there are no significant differences between intact and GDX naked mole-rats of both sexes in chapter 3 of this thesis. Thus, the rapid onset of fertility observed when subordinate naked and Damaraland mole-rats are released from socially-induced reproductive suppression may be entirely independent from gonadal hormone control. These results suggest that independence from gonadal control may be a hallmark of eusociality. It would be interesting to see whether other social animals like marmoset monkeys and other social mole-rats also have neuroendocrine systems that are relatively independent from gonadal steroids.

There is a lack of significant differences in the number of MPOA RFamide-ir cell bodies between male and female Damaraland mole-rats. Despite the significant differences observed between the breeders and subordinates, breeding males do not differ from breeding females, and subordinate males do not differ from subordinate females in their number of MPOA RFamide-ir cell bodies. Interestingly, in chapter 3 of this thesis I have described how naked mole-rats also have a sexually monomorphic kisspeptin system whereby both females and males have very similar numbers of kisspeptin-ir cells in the RP3V/PVH region. Furthermore, naked mole-rats also show a lack of sexual differentiation in their external genitalia, behaviour and neuroanatomy, instead, these biological aspects may be determined by the social status that individuals hold within a colony (Holmes *et al.*, 2009). These findings indicate that the neurobiology of naked and Damaraland mole-rats are both relatively sexually monomorphic and independent from gonadal hormones, compared to traditional laboratory rodents. Moreover, both of these characteristics may be unique to eusocial animals.

The data from this present study reveals that male breeding Damaraland mole-rats are significantly heavier than female breeders and subordinate of both sexes. This result is not so surprising given that previous studies have shown sex differences and breeding status differences in body mass (Bennett *et al.*, 1993; Anyan *et al.*, 2011). Also, there is a positive correlation between body weight and the number of MPOA RFamide-ir cell bodies in Damaraland mole-rats. This is interesting since previous studies have shown that there is a positive correlation between body mass and the

magnitude to the response of a GnRH challenge (Bennett *et al.*, 1993). Hence the heavier Damaraland mole-rats have a greater magnitude of gonadotrophin release, and a greater number of RFamide-ir cell bodies in the MPOA.

In chapter 3 of this thesis, I have found no significant correlation between body mass and the number of kisspeptin-ir cell bodies in the RP3V or PVH of naked mole-rats. Whilst male breeding Damaraland mole-rats are significantly heavier than all other female groups, there is no effect of sex on the body weight of naked mole-rats. This difference between naked and Damaraland mole-rats may indicate the absence of the effects of sex in the former and the presence of the effects of sex in the latter species. Other researchers have also concluded that naked mole-rats have no sexually differentiated brain regions, whilst Damaraland mole-rats have significant sex differences in certain brain regions, such as the MeA and the Onuf's nucleus (Holmes *et al.*, 2009; Anyan *et al.*, 2011). So whilst neural dimorphisms are associated with breeding status in naked mole-rats, the neural dimorphisms in Damaraland mole-rats may be associated with both sex and breeding status. Therefore, whilst both naked and Damaraland mole-rats are relatively independent from gonadal hormones, the effects of sexual differentiation seem to have a much greater effect on the neurobiology and physiology of Damaraland mole-rats than naked mole-rats. Whether this phenomenon is related to the species-specific differences in reproductive skew remains to be answered. Moreover, the fact that Damaraland mole-rats can be independent from gonadal steroids and simultaneously show aspects of sexual differentiation in their neurobiology, automatically poses the question as to what the mechanisms behind this sexual differentiation actually are.

CHAPTER 5:

Analysis of tyrosine hydroxylase neuronal systems in the diencephalon of the eusocial naked mole-rat (*Heterocephalus glaber*)

ABSTRACT

Naked mole-rats are remarkably sexually monomorphic in many aspects of their behaviour, physiology and neuroanatomy. I have previously reported the lack of sexual differentiation in the kisspeptin system in the brains of naked mole-rats− a neuropeptide system which is sexually dimorphic in typical laboratory rodents. Expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for the synthesis of dopamine and other catecholamines, is found in many areas of the rodent brain. In rats and mice, the TH-expressing cell population in the anteroventral periventricular nucleus (AVPV) is sexually differentiated, containing two to four times more TH expressing cells in females than males. Here, I mapped TH-immunoreactivity and examined sex differences in TH-immunoreactive (ir) cell populations in the diencephalon of eusocial naked mole-rats, a species that shows unusual sexual monomorphism. TH-ir cell bodies were located in the rostral periventricular region of the third ventricle (RP3V) that consists of the AVPV and periventricular nucleus,, anterior hypothalamic nucleus (AHN), bed nucleus of the stria terminalis (BNST), paraventricular hypothalamic nucleus (PVH), the zona incerta (ZI), the arcuate nucleus (Arc) and regions of the thalamus. There was a low density of TH-ir cells in the RP3V, AHN, BNST and Arc. The largest populations of TH-ir cell bodies were found in the PVH and ZI. There was a lack of sexual differentiation in the numbers of TH-ir cell bodies in all brain regions investigated. The findings of this study further emphasise the sexual monomorphism of these eusocial animals.

INTRODUCTION

Expression of TH, the rate-limiting enzyme for the synthesis of dopamine and other catecholamines, is found in many areas of the rodent brain. Very dense populations of TH-synthesising dopaminergic cells are located in the ventral tegmental area (VTA), substantia nigra pars compacta (SNpc) and the hypothalamus (Bjorklund *et al.*, 1975; Chan-Palay *et al.*, 1984; Lansing and Lonstein, 2006). TH-expressing cells are also found in the hypothalamus of other species, such as amphibians, reptiles and birds, indicating that this TH system is conserved in vertebrates (Smeets and Gonzalez, 2000). In rats and mice, the TH-expressing cells are dopaminergic and this cell population in the AVPV is sexually differentiated, containing two to four times more TH-expressing cells in females than males (Simerly *et al.*, 1985a; Simerly *et al.*, 1985b; Simerly, 1989; Simerly *et al.*, 1997).

The magnitude of this sex difference in the number of TH-expressing cells in the rat AVPV is influenced by adult circulating gonadal hormones. Castration of males significantly increases TH mRNA expression and TH-immunoreactivity in the AVPV. In females, ovariectomy has no effect on TH expression, however, chronic treatment with high levels of oestradiol reduces TH expression in the AVPV (Simerly *et al.*, 1985b; Simerly, 1989). Similarly, treatment of adult male rats with high levels of testosterone downregulates TH expression in the AVPV (Simerly, 1989). In addition to the effect of adult circulating gonadal hormones, the sex difference in rats is eliminated by castrating males on the day of birth and treating the neonatal females with testosterone (Simerly, 1989), suggesting that perinatal gonadal steroids influence the number of TH-expressing cells in the AVPV.

In this thesis, I have previously discussed the sexually differentiated population *Kiss1* neurones in the AVPV of mice and rats- where adult females have many more cells than males (see chapter 3). Since both kisspeptin and TH neuronal populations are located in the AVPV, recent studies have investigated whether these cells belong to the same neuronal population or whether they are separate sexually dimorphic systems located in the same brain region. In adult female rats, the vast majority of AVPV/PeN *Kiss1* neurones do not co-express *TH* mRNA, and the few *Kiss1* neurones that are co-labelled express only low levels of *TH* mRNA (Kauffman *et al.*, 2007). Therefore, despite some overlap in the anatomic distribution of these two neuronal populations, the sexually dimorphic *Kiss1* and *TH* populations in the AVPV/PeN appear to comprise two separate, sexually-differentiated populations with only a minor degree of overlap.

Naked mole-rats are remarkably sexually monomorphic in aspects of their neuroanatomy (Holmes *et al.*, 2009). In chapter 3 of this thesis, I discovered that there

were no differences in the number of kisspeptin-ir cells in the RP3V or PVH between female and male naked mole-rats. In this present study, our aim was to (1) map the distribution of TH-immunoreactivity in the brains of naked mole-rats, and (2) determine whether the TH neuronal system in naked mole-rats is sexually differentiated.

MATERIALS AND METHOD

Study animals

The current experiment compared 2 groups of naked mole-rats: (group 1) female subordinates (N= 6), and (group 2) male subordinates (N= 6).

Tyrosine hydroxylase immunohistochemistry

One series of sections was immunostained for tyrosine hydroxylase (polyclonal rabbit anti-tyrosine hydroxylase 1:40,000; Sigma-Aldrich, Dorset, UK) as described above in chapter 3 for GnRH-1 immunohistochemistry. The secondary antibody used was biotinylated donkey anti-rabbit IgG (1:1000; Stratech, Newmarket, Suffolk, UK).

Digital photomicrographs

Brightfield photomicrographs were obtained using a Nikon E600 microscope at magnifications of x40, x100 and x200 with a Micro-Publisher 5.0 camera (InterFocus Imaging, Cambridge, UK). The camera was controlled by MCID Core software (Interfocus Imaging). Images were later post-processed using Adobe Photoshop, CS3 to adjust brightness and contrast. Background artefacts were removed as necessary; no other modifications were made to images. Final images were compiled into multi-panel plates in Microsoft Publisher, minor changes to brightness and contrast were made after importing into Microsoft Publisher if necessary.

Quantification and statistical analysis

To compare the presence of TH-immunoreactivity in the brains of naked mole-rats, the entire rostrocaudal extents of the RP3V (consisting of the AVPV and PeN), the anterior hypothalamus (AH), the BNST, the PVH and the zona incerta (ZI) were examined for each subject. Slides were masked and coded for analysis. For each animal, the number of TH-ir cell bodies in the RP3V, AHN, BNST, PVH and ZI were counted by eye with a Nikon E600 microscope at X200 magnification. TH-ir cell bodies were counted in each rostro-caudal section and added together. The number of TH-ir cell bodies was multiplied by 6 to obtain to the total number of cells in the brains of each animal. TH-ir cell bodies were not quantified in the reuniens thalamic nucleus (Re) or Arc due to significant tissue damage. For all comparisons, statistical significance was set at $p < 0.05$. An independent samples T-test was undertaken to determine whether there were significant differences between the number of TH-ir cells bodies in the RP3V, AH, BNST, PVH and ZI of female and male naked mole-rats.

RESULTS

The distribution of TH-ir cell bodies

TH-ir cell bodies were located in the RP3V, BNST, AHN, PVH, ZI, Arc and Re (Plate 5.1A1-D2). A diffuse scatter of TH-ir cell bodies were observed in the AHN and BNST (Plate 5.1A1-A2). TH-ir cell bodies were loosely scattered along the midline in the RP3V, with some cells extending laterally (Plate 5.1B1-B2). There was a dense cluster of TH-ir cell bodies scattered throughout the PVH, with a few cells extending laterally (Plate 5.1C1-C2). More caudally, there was a light scatter of TH-ir cell bodies in the Arc, VMH and DMH located close to the 3V (Plate 5.1D1-D2). There was a distinct absence of TH-ir cell bodies in the SCN (Plate 5.1C1). The largest populations of TH-ir cell bodies were observed in the ZI and Re (Plate 5.2). In the ZI, a few TH-ir cells bodies extended medially towards the midline and another cluster of TH-ir cell bodies extended dorsally encircling the mammillothalamic tract (mt) (Plate 5.2). The dense cluster of TH-ir cell bodies in the Re extended dorsally towards the paraventricular thalamic nucleus (PVT) (Plate 5.2). Finally, there was a light scatter of TH-ir cell bodies located throughout the MM at the base of the hypothalamus.

The distribution of TH-ir processes

In the rostral forebrain, abundant TH-ir processes were observed throughout the striatum. A few, sparse TH-ir processes were observed in the AHN, the lateral hypothalamus and in the BNST (Plate 5.1A1-A2). In the RP3V (consisting of the AVPV and PeN), sparse bundles of TH-ir processes were observed in the midline in the dorsoventral orientation, in the vicinity of TH-ir cell bodies (Plate 5.1B1-B2). In the PVH, dense of TH-ir processes formed an inverted triangle shaped clusters of fibres (Plate 5.1C1-C2). Dorsally, clusters of TH-ir processes were orientated in the medio-lateral orientation, and extended into the internal capsule (ic) (Plate 5.1C1). A few TH-ir processes were located in the vestigial OCh. There was a distinct absence of TH-ir processes in the SCN. More caudally, TH-ir processes were located in the Arc and DMH (Plate 5.1D1-D2). There was a light scatter of TH-ir processes in the MM at the base of the hypothalamus. Dorsally in the region of the thalamus, there was a dense cluster of TH-ir cells in the ZI (Plate 5.2). From the ZI, TH-ir processes extended ventrally into the ic (Plate 5.2). In the medial ZI, a cluster of TH-ir processes extended upwards and encircled the mt (Plate 5.2). Another dense cluster of TH-ir processes were located in the Re close to the midline, extending into the PVT (Plate 5.2).

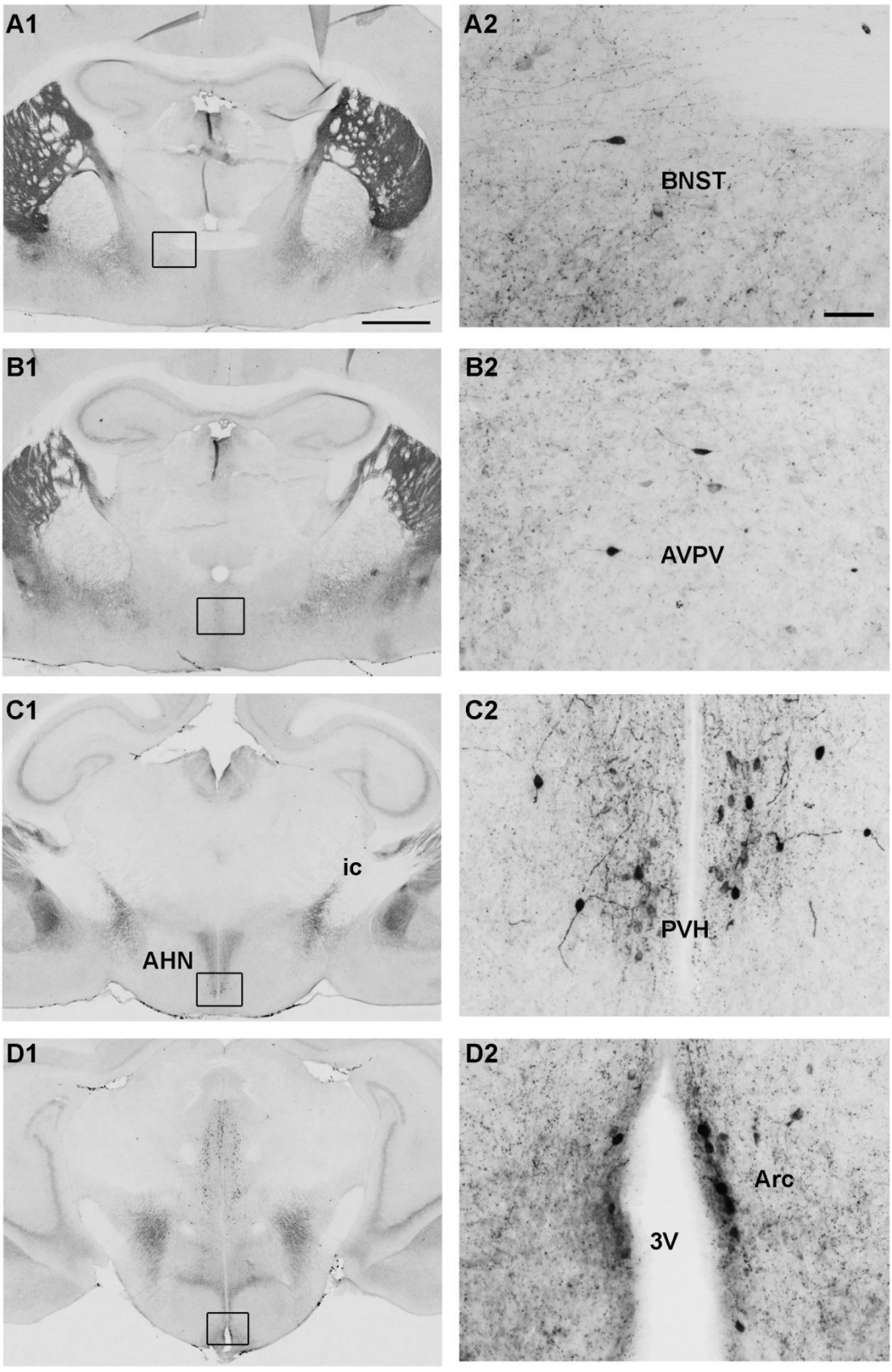


Plate 5.1: Brightfield photomicrographs of representative coronal sections (A1-D1; rostral to caudal) showing tyrosine hydroxylase-immunoreactivity in the brain of a subordinate female naked mole-rat. Areas enclosed by a box on the left are shown in higher magnification on the corresponding right. 3V, third ventricle; AHN, anterior hypothalamic nucleus; Arc, arcuate

nucleus; AVPV, anteroventral periventricular nucleus; BNST, bed nucleus of the stria terminalis; ic, internal capsule; PVH, paraventricular hypothalamic nucleus. Scale bars= 1mm for A1, B1, C1 and D1; 50µm for A2, B2, C2 and D2.

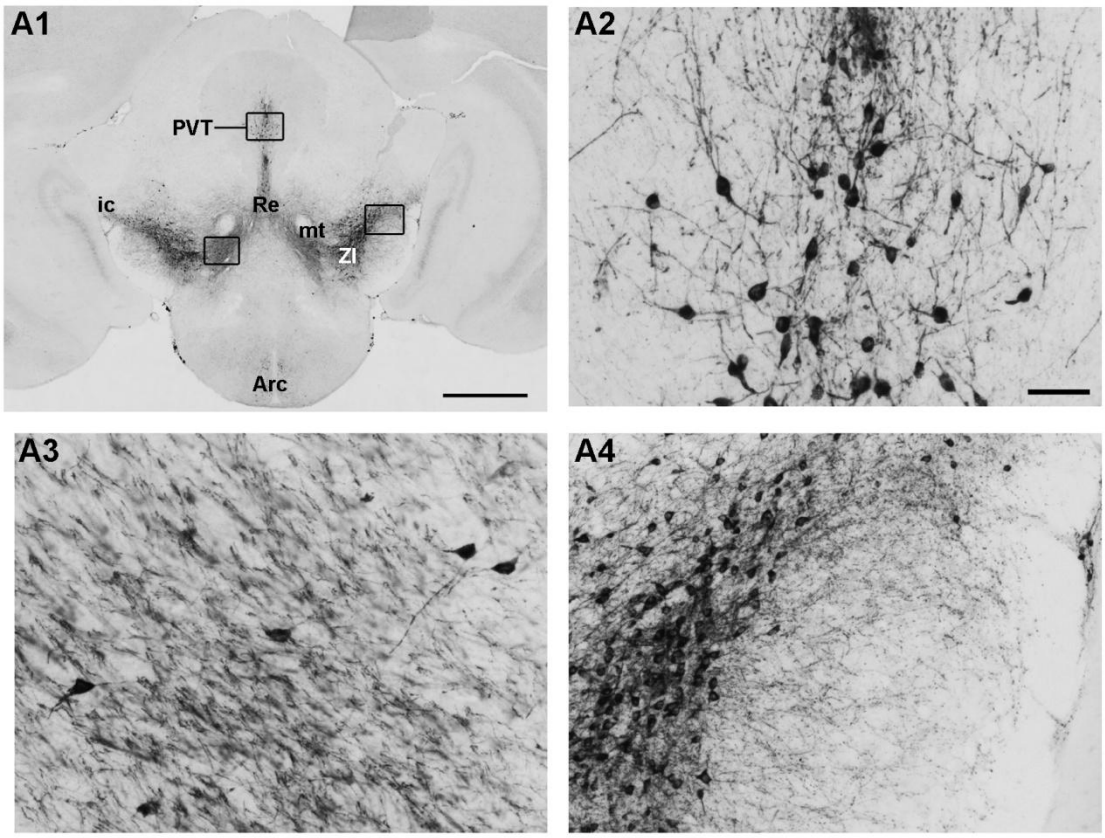


Plate 5.2: Brightfield photomicrographs of representative coronal sections showing tyrosine hydroxylase-immunoreactivity in the diencephalon of a subordinate female naked mole-rat. In A1, the top area enclosed by a box is shown in higher magnification in A2. In A1, the bottom left area enclosed by a box is shown in higher magnification in A3. In A1, the bottom right area enclosed by a box is shown in higher magnification in A4. Arc, arcuate nucleus; ic, internal capsule; mt, mammillothalamic tract; PVT, paraventricular thalamic nucleus; Re, reuniens thalamic nucleus; ZI, zona incerta. Scale bar= 1mm for A1; 50µm for A2, A3 and A4.

The number of TH-ir cell bodies

There were no significant differences in the number of TH-ir cell bodies between females and males in the RP3V ($t(10)= 0.115$, $p= 0.911$), AHN ($t(10)= 0.320$, $p= 0.756$), BNST ($t(10)= 0.496$, $p= 0.630$), PVH ($t(10)= 0.923$, $p= 0.378$) or ZI ($t(10)= 0.238$, $p=0.817$) (Figure 5.1). Interestingly, the PVH of both sexes contained the largest number of TH-ir cell bodies compared to other hypothalamic regions (Figure 5.1).

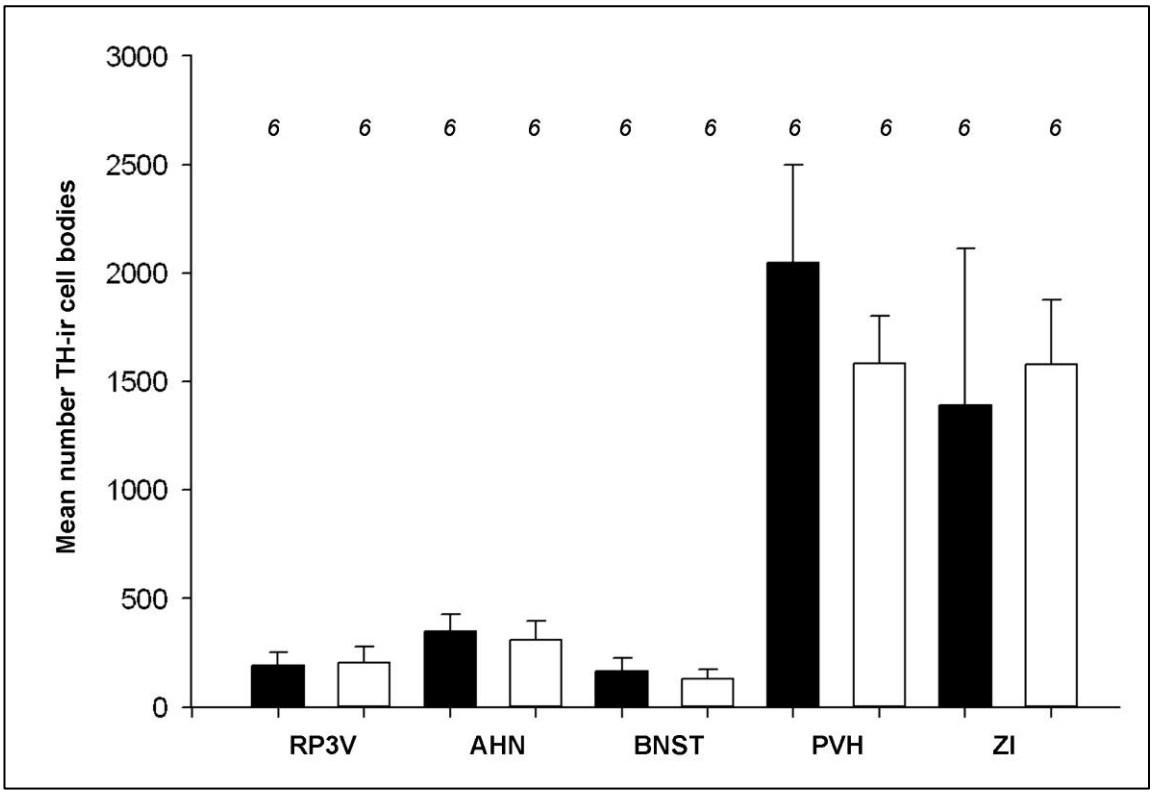


Figure 5.1: Mean (± SEM) number of tyrosine hydroxylase-ir cell bodies in the rostral periventricular region of the third ventricle (RP3V), anterior hypothalamic nucleus (AHN), bed nucleus of the stria terminalis (BNST), paraventricular hypothalamic nucleus (PVH) and zona incerta (ZI). Number of animals per group is noted at the top in italics. Black bars represent females; open bars represent males.

DISCUSSION

The present results are the first to demonstrate that the hypothalamus and surrounding regions of the naked mole-rat brain contain numerous populations of neurones expressing TH. In rats, these cells are very likely to be dopaminergic (Simerly *et al.*, 1985a; Simerly *et al.*, 1985b). The function of these hypothalamic TH cell groups in naked mole-rats is unknown, but may be involved in functions similar to those in rats, including prolaction (Ben-Jonathan and Hnasko, 2001) and gonadotrophin release (Simerly *et al.*, 1985a; Simerly *et al.*, 1985b). In rats and mice, the TH-expressing cell population in the AVPV is sexually differentiated, containing two to four times more TH expressing cells in females than males (Simerly *et al.*, 1985a; Simerly *et al.*, 1985b; Simerly, 1989; Simerly *et al.*, 1997). The RP3V region contains a large population of sexually differentiated *Kiss1* neurones, with female mice and rats containing a significantly larger number of *Kiss1* neurones (Kauffman *et al.*, 2007; Clarkson *et al.*, 2009a; Kauffman *et al.*, 2009). RP3V kisspeptin neurones project to GnRH neurones and have been shown to mediate the oestradiol-induced positive feedback that leads to a GnRH/LH surge (Kauffman, 2010).

An *in situ* hybridisation study in rats has shown that the vast majority of RP3V *Kiss1* neurones do not co-express *TH* mRNA, and that *Kiss1* and TH populations in the RP3V are likely to be separate populations within the RP3V (Kauffman *et al.*, 2007). However, a more recent study investigating the association between kisspeptin- and TH-ir cells has shown that half of the kisspeptin neurones in the RP3V express TH and vice versa, and a quarter exhibited a close apposition from a TH-ir fibre (Clarkson and Herbison, 2011). Furthermore, many of the TH close appositions with GnRH neurones coexpressed kisspeptin, indicating that a sub-population of RP3V kisspeptin neurones in female mice are likely to utilise both kisspeptin and dopamine as neurotransmitters (Clarkson and Herbison, 2011). Electron microscopic studies in the rat have provided evidence that TH-containing inputs synapse on GnRH neurones, suggesting that they are functional (Chen *et al.*, 1989). Another study indicated that AVPV dopamine neurones project to GnRH neurones in the rat. Hence, one role of the RP3V dopamine neurones may involve the sexually differentiated regulation of GnRH neurones (Horvath *et al.*, 1993). These studies suggest that the RP3V is an important source of dopamine input to GnRH neurones. However, the catecholamine innervations of the hypothalamus are very complex since noradrenergic, dopaminergic and adrenergic axons all contain the enzyme TH. Thus, in the future it will be important to separate the effects of these three catecholamine systems in the hypothalamus

The distribution of TH-immunoreactivity

The distribution of TH-immunoreactivity in the diencephalon of naked mole-rats is similar to that observed in other rodents (Chan-Palay *et al.*, 1984; Simerly *et al.*, 1997), however, there are some species specific differences. Like most rodents, TH-ir cell bodies are located in the RP3V, BNST, PVH, AHN, Arc, ZI and Re. Intriguingly, most of the TH-ir cell bodies were located in the PVH, ZI and RE of naked mole-rats. On the contrary, TH-immunoreactivity was very sparse in the rest of the hypothalamus. In the monogamous prairie vole, there was a high density of TH-ir cell bodies in the AVPV, Arc, BNST and MeA (Lansing and Lonstein, 2006; Northcutt *et al.*, 2007). In a sex and species comparison of TH-immunoreactivity, male prairie voles had a large number of TH-ir cell bodies in the BNST and MeA, however, female prairie voles, meadow voles, hamsters and rats of both sexes had relatively few or no TH-ir cell bodies at these sites (Northcutt *et al.*, 2007). In this respect, TH-immunoreactivity in the BNST of naked mole-rats is similar to most other rodents, apart from the male prairie vole. Interestingly, castration reduced the number of TH-ir cell bodies in male prairie voles, an effect that can be prevented by chronic treatment with testosterone. Female prairie voles treated with testosterone during adulthood show a significant increase in the number of TH-ir cell bodies in the BNST (Northcutt *et al.*, 2007). Although the contrasting TH-immunoreactivity between male prairie and meadow voles may be explained by their differences in social organisation, with the prairie voles being monogamous and meadow voles being promiscuous, the eusocial male naked mole-rats (whilst they are not technically monogamous, the male and female breeding pairs do form life long bonds) have relatively few TH-ir cell bodies in the BNST. This suggests that a high TH-immunoreactivity in the BNST is specific to just male prairie voles and is not typical in the males of other social species.

There was a distinct absence of TH-immunoreactivity in the SCN. In the vestigial OCh and opt, there was only a slight scatter of TH-ir processes. These results are perhaps not so surprising given that naked mole-rats are almost blind, living a subterranean lifestyle and have a vestigial accessory optic system (Crish *et al.*, 2006). Similarly, there is no kisspeptin- or GnRH-immunoreactivity in the SCN of naked mole-rats (see chapter 3). Interestingly, naked mole-rats have extensive TH-innervation of the thalamus. Naked mole-rats have an unusually high density of TH-ir cell bodies in the Re which extends dorsally into the PVT nucleus. However, the function of these extrahypothalamic TH-ir cell bodies is not known.

The number of TH-ir cell bodies

Whilst the number of TH-ir cell bodies was generally low in the hypothalamus, naked mole-rats of both sexes had a very high number of TH-ir cell bodies in the PVH and ZI.

The PVH is thought to play a role in the reproductive-activation of subordinate naked mole-rats since this area contains a high number of kisspeptin-ir cell bodies and breeding/reproductively-activated naked mole-rats have a significantly higher number of kisspeptin-ir cell bodies in the PVH than subordinates (refer to chapter 3). It remains an intriguing fact that the PVH in naked mole-rats contains dense populations of both kisspeptin- and TH-ir cell bodies. Although it is not known whether these TH-ir neurones have any relationship with kisspeptin-ir neurones in the PVH, further double-labelling studies will help us to determine whether there are any terminal contacts between the kisspeptin- and TH-ir neurones in the PVH.

There was no significant difference in the number of TH-ir cell bodies in all brain regions investigated. Whilst sexually differentiated populations of hypothalamic neurones have been demonstrated in rats, mice and prarie voles (Simerly *et al.*, 1985a; Simerly *et al.*, 1985b; Simerly, 1989; Simerly *et al.*, 1997; Lansing and Lonstein, 2006; Kauffman *et al.*, 2007; Clarkson *et al.*, 2009b), results of this study and chapter 3 confirm that naked mole-rats have a sexually monomorphic hypothalamus. Instead, aspects of naked mole-rat neurobiology are thought to be dictated by their social/reproductive status (Holmes *et al.*, 2009). Chapter 3 of this thesis showed that the number of kisspeptin-ir cell bodies in the RP3V and PVH of naked mole-rats are sexually monomorphic

In future studies, it would be interesting to investigate TH-immunoreactivity in different species of mole-rats with different social structures to determine whether there are any effects of sex or social status on TH-immunoreactivity. It would also be interesting to compare TH-immunoreactivity between breeding and subordinate naked mole-rats, intact and GDX, to determine whether reproductive-activation or gonadal hormones have an effect of the TH neuronal system in naked mole-rats.

CHAPTER 6:

Distribution of oxytocin and vasopressin and their binding sites in eusocial Damaraland mole-rats (*Fukomys damarensis*): implications for eusocial behaviour

ABSTRACT

Mole-rats (Bathyergidae family) provide a unique taxonomic group for studying the neurobiology of sociality. Ecological constraints have led to diverse social and reproductive strategies in mole-rats. Studies on *Microtus* voles have implicated telencephalic oxytocin and vasopressin binding sites in social recognition, monogamy and alloparental behaviour. Previous studies have shown that eusocial naked mole-rats display high levels of oxytocin and its receptor (OTR) binding in the nucleus accumbens (NAcc). Both factors are lacking in the NAcc of solitary Cape mole-rats. Vasopressin receptor (V1aR) binding is present in the ventral pallidum (VP) of solitary Cape mole-rats, but is absent from the VP of eusocial naked mole-rats. Despite extreme differences in sociality, both show septal V1aR binding. This study examined the distribution of oxytocin and vasopressin, and their binding sites in eusocial Damaraland mole-rats. OTR binding was intense in the NAcc and Islands of Calleja. Oxytocin-immunoreactive (ir) processes were found in the NAcc, but not in the other sites. V1aR binding was absent from the VP of Damaraland mole-rats and vasopressin-ir processes were present in the NAcc of Damaralands. The V1aR binding was intense in the NAcc and anteroventral periventricular nucleus (AVPV) of Damaralands. Therefore, one can conclude that: (1) the presence of oxytocin and its receptor in the NAcc of both eusocial mole-rats may be an example of convergent evolution within the Bathyergidae family, and this may confer sociality, (2) the absence of vasopressin and its receptor V1aR in the VP of both eusocial mole-rats may indicate a loss of function in the Bathyergidae family, (3) given the evidence that eusociality has evolved twice in the family Bathyergidae, it is possible that the origins of Damaraland eusociality may relate to the parallel emergence of V1aR and OTR and their respective ligands in the NAcc and (4) the emergence of V1aR in the AVPV in eusocial Damaraland mole-rats may have evolved independently as these animals diverged from the common ancestor.

INTRODUCTION

African mole-rats (family Bathyergidae) provide a unique taxonomic group for studying the neurobiology of sociality. Ecological constraints have led to diverse social and reproductive strategies within this family (Faulkes *et al.*, 1997). Damaraland mole-rats exhibit an extreme form of socially induced infertility, in which only one dominant female and one or two males within a colony breed; the subordinates fail to breed (Bennett and Jarvis, 1988; Burland *et al.*, 2004). Studies on *Microtus* voles have implicated telencephalic OT and AVP in social recognition, monogamy and alloparental behaviour. Previous studies compared the only other eusocial member of this family, naked mole-rats, with solitary Cape mole-rats (Kalamatianos *et al.*, 2010). Naked mole-rats show high levels of OT and OTR binding in the NAcc. In Cape mole-rats, this site is devoid not only of this peptide, but also of its binding signals. In contrast, Cape mole-rats have high levels of V1aR binding in the VP, but there is no such binding in naked mole-rats. Neither species shows AVP-immunoreactivity in the VP. Despite their extreme differences in sociality, each of those species shows AVP and V1aR binding in the LS.

In this present study, I investigated (a) the distribution of OT- and AVP-ir cell bodies and processes, and (b) the telencephalic distribution of OTR and V1aR in the eusocial Damaraland mole-rat brain. This research is contributing to our long-term aim of elucidating the neurobiology and molecular biological regulation of OT and AVP systems and their evolution across the spectrum of eusocial, social and solitary mole-rat species.

MATERIALS AND METHOD

Study animals

The OT and AVP immunohistochemistry experiment compared 2 groups of Damaraland mole-rats: (group 1) female subordinates (N= 5), and (group 2) male subordinates (N= 5). The OTR and V1aR ligand binding study analysed 1 group of Damaraland mole-rats: (group 1) female subordinates (N= 5). Only subordinate animals were investigated since these animals had lower levels of circulating gonadal steroids (Bennett *et al.*, 1993; Bennett, 1994; Bennett *et al.*, 1996; Molteno and Bennett, 2000).

Oxytocin and vasopressin immunohistochemistry

One series of sections was immunostained for OT-associated neurophysin (monoclonal mouse anti-OT-associated neurophysin 1:500; PS-38 gift from H. Gainer) and another series was immunostained for AVP (polyclonal rabbit anti-AVP 1:20,000; ICN Biomedicals, Aurora, OH, USA) as described above in chapter 3 for GnRH-1 immunohistochemistry (minus the treatment with 0.1% sodium borohydride). The secondary antibody used was biotinylated donkey anti-mouse IgG (for mouse antibody) or biotinylated donkey anti-rabbit IgG (for rabbit antibody) (1:1000; Stratech, Newmarket, Suffolk, UK). The OT-associated neurophysin antibody has previously been characterised (Ben-Barak *et al.*, 1985; Whitnall *et al.*, 1985). The mouse OT-associated neurophysin was raised against extracts of rat posterior pituitary coupled to keyhole limpet hemocyanin. In immunoprecipitation assays, the OT-associated neurophysin antibody bound the appropriate neurophysin as well as their precursor molecules with no cross-reactivity. For the AVP antibody, the antigen used in the production of this antibody was synthetic full length AVP (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2) bound to bovine thyroglobulin with carbodiimide.

OTR and V1aR ligand binding autoradiography

Brains were cut into serial coronal sections (20 µm) with a cryostat (Bright Cryostats, UK). Two adjacent sections were thaw-mounted onto Superfrost Plus slides (Sigma-Aldrich, Dorset, UK) in four rostrocaudal series for each animal. They were allowed to air dry at room temperature and stored at –80°C until use. One set of slides were processed for OTR autoradiography using a selective ^{125}I-labelled OTR antagonist [^{125}I]-ornithine vasotocin analogue (d[CH_2]$_5$[Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]-vasotocin; 2200 Ci/mmol; Perkin Elmer, Boston, MA). The second set of slides were processed for V1aR autoradiography using ^{125}I-labelled V1aR antagonist ^{125}Id(CH_2)$_5$(Tyr[Me])-AVP (PerkinElmer, Boston, MA). Briefly, slides were brought to room temperature and

immersed in freshly prepared 0.1% PFA in 0.1 M PBS (pH 7.4) for 2.5 minutes. Sections were rinsed twice in Tris-HCl buffer (pH 7.4) and incubated for 120 minutes at room temperature in 50 pM of either ^{125}I-OTR antagonist or ^{125}I-V1aR antagonist in Tris-HCl buffer (pH 7.4) containing 10 mM MgCl2, 0.1% bovine serum albumin (Sigma-Aldrich), and 0.05% bacitracin (Sigma-Aldrich). Unbound ligand was removed by four 5-minute washes in cold Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, followed by a quick rinse in H_2O. Sections were finally dried under a stream of cold air prior to exposure to BioMax MR Film (Kodak, Rochester, NY) along with ^{125}I autoradiographic microscale standards (GE HealthCare, Bucks, UK) for 5 days. In an adjacent series of sections, competition of ^{125}I-OTR antagonist binding with 1 µM nonradioactive oxytocin (Sigma-Aldrich) and competition of ^{125}I-V1aR antagonist binding with 1 µM nonradioactive arginine vasopressin (Sigma-Aldrich) resulted in loss of autoradiographic signals. After film development, sections were Nissl stained (0.5% cresyl violet acetate), dehydrated, and coverslipped for microscopic examination.

Digital photomicrographs

Brightfield photomicrographs were obtained using a Nikon E600 microscope at magnifications of x40, x100 and x200 with a Micro-Publisher 5.0 camera (InterFocus Imaging, Cambridge, UK). Darkfield photomicrographs were taken at x20 and x40. Photomicrographs of Nissl stained sections were taken using a precision illuminator (Northern Light model R95; Interfocus Imaging) with a CoolSnap CF camera (Photometrics, Marlow, UK). Both cameras were controlled by MCID Core software (Interfocus Imaging). Images were later post-processed using Adobe Photoshop, CS3 to adjust brightness and contrast. Background artefacts were removed as necessary; no other modifications were made to images. Final images were compiled into multi-panel plates in Microsoft Publisher, minor changes to brightness and contrast were made after importing into Microsoft Publisher if necessary.

Only photomicrographs of females subordinates are presented since which they are indistinguishable from a male subordinates.

RESULTS

Distribution of telencephalic OXTR and V1aR binding

A high level of OTR binding was observed in the NAcc and Islands of Calleja (ICj) of female Damaraland mole-rats (Plate 6.1A2, B2 and C2). There was no OTR binding in the LS, the VP or the BNST (Plate 6.1A2, B2, C2 and D2). A high level of V1aR binding was observed in the NAcc (Plate 6.2B2) and AVPV (Plate 6.2D2), and a low level of V1aR binding was observed in the LS (Plate 6.2C2) of female Damaraland mole-rats. There was no V1aR binding in the VP or BNST of female Damaraland mole-rats.

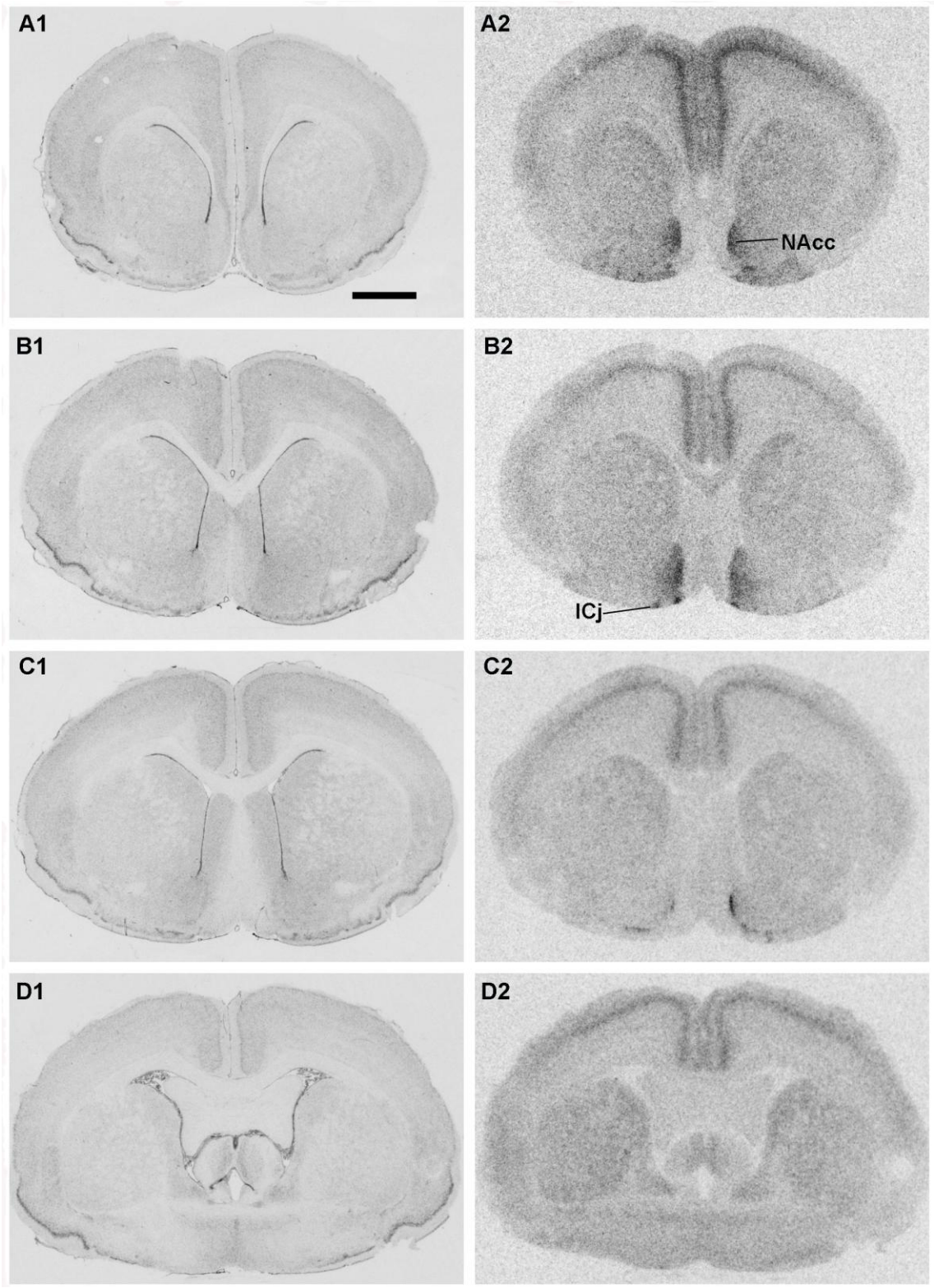


Plate 6.1: Brightfield photomicrographs of representative Nissl-stained coronal sections (A1, B1, C1 and D1) and corresponding film autoradiographs (A2, B2, C2 and D2) showing oxytocin receptor (OTR) binding at rostrocaudal levels of a female subordinate Damaraland mole-rat. Nucleus accumbens (NAcc) and Islands of Calleja (ICj). Scale bar= 2mm.

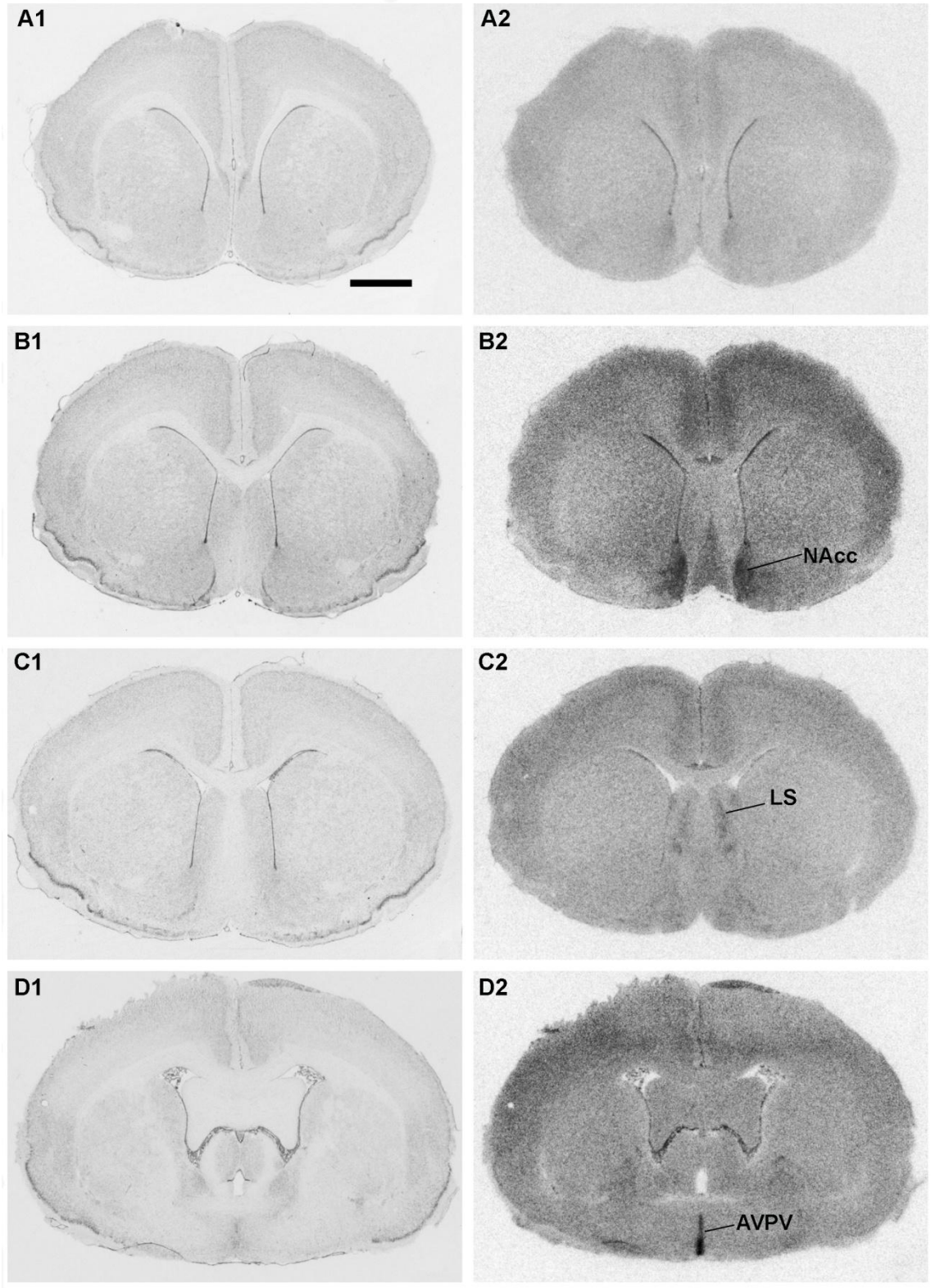


Plate 6.2: Brightfield photomicrographs of representative Nissl-stained coronal sections (A1, B1, C1 and D1) and corresponding film autoradiographs (A2, B2, C2 and D2) showing vasopressin receptor (V1aR) binding at rostrocaudal levels of a female subordinate Damaraland mole-rat. Nucleus accumbens (NAcc), lateral septum (LS) and anteroventral periventricular hypothalamic nucleus (AVPV). Scale bar= 2mm.

Distribution of OT-neurophysin-ir cell bodies

The majority of the OT-neurophysin-ir cell bodies were located in the PVH and SON (Plate 6.3A1). These OT-neurophysin-ir cell bodies were darkly stained and spread throughout the PVH (Plate 6.3A2). Most of the OT-neurophysin-ir cell bodies were located on the lateral margins of the PVH, with few located near the lining of the 3V (Plate 6.3A1). In the rostral PVH, OT-neurophysin-ir cell bodies were distributed near the midline surrounding the 3V in a dorso-ventral orientation. Scattered OT-neurophysin-ir cell bodies were distributed ventral to the base of the PVH (Plate 6.3A1). At the level of the rostral PVH, OT-neurophysin-ir cell bodies were also located along the vestigial optic tract (opt) in the vicinity of the OCh. Within the SON, dense clusters of OT-neurophysin-ir cell bodies were distributed medio-laterally, extending into the optic tract (Plate 6.3A3). A few scattered OT-neurophysin-ir cell bodies were located in the lateral hypothalamus.

Distribution of hypothalamic OT-neurophysin-ir processes

Within the hypothalamus, OT-neurophysin-ir processes were located in the PVH and SON (Plate 6.3A1). Some of the OT-neurophysin-ir cell bodies in the PVH sent processes medially towards the 3V (Plate 6.3A1). The majority of OT-neurophysin-ir processes from the PVH and SON projected towards and through the internal zone of the ME (Plate 6.3B1-B2). The OT-neurophysin-ir processes formed a tight arching band within the ventral aspect of the internal zone of the ME (Plate 6.3B1-B2)− where the immunoreactivity was particularly dense. There were very few OT-neurophysin-ir processes in the external zone of the ME (Plate 6.3B2). In the rostral PVH, OT-neurophysin-ir processes were distributed along the vestigial opt and OCh medio-laterally along the base of the hypothalamus (Plate 6.3A1). These rostrally occurring OT-neurophysin-ir processes extended into the AVPV (Plate 6.4A1-A2), surrounding the midline and 3V in the dorso-ventral orientation. There were also some scattered OT-neurophysin-ir processes in the lateral hypothalamus. No OT-neurophysin-ir processes were observed in the SCN.

Distribution of extrahypothalamic OT-neurophysin-ir processes

OT-neurophysin-ir processes were also distributed in many extra-hypothalamic areas. There were very few (almost negligible) OT-neurophysin-ir processes present in the MeA (Plate 6.4B1-B2). In the forebrain, small clusters of OT-neurophysin-ir processes were found in the intermediate part of the LS− these OT-neurophysin-ir processes did not extend towards the LV nor did they extend towards the dorsal aspect of LS (Plate 6.5B1-B2). Diffuse networks of OT-neurophysin-ir processes were located in the NAcc (Plate 6.5A1-A2) and BNST (Plate 6.5C1-C3).

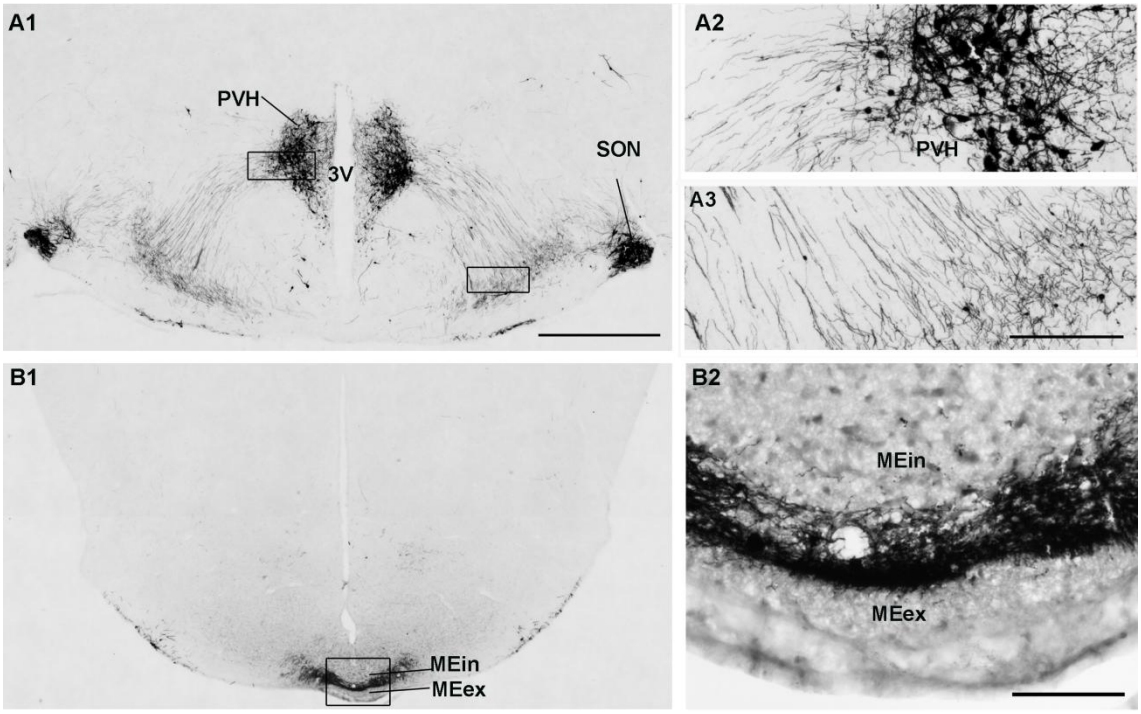


Plate 6.3: Brightfield photomicrographs of representative coronal sections showing: (A1-A3) oxytocin-neurophysin-immunoreactive cell bodies within the paraventricular hypothalamic nucleus and supraoptic nucleus, and (B1-B2) oxytocin-neurophysin-immunoreactive processes within the median eminence of a female subordinate Damaraland mole-rat. Areas enclosed by a box (A1, B2) are shown in higher magnification (A2-A3, B2). 3V, third ventricle; PVH, paraventricular hypothalamic nucleus; MEex, external zone of the median eminence; MEin, internal zone of the median eminence and SON, supraoptic nucleus. Scale bars= 100µm for A1 and B1; 20µm for A2 and A3; 10µm for B2.

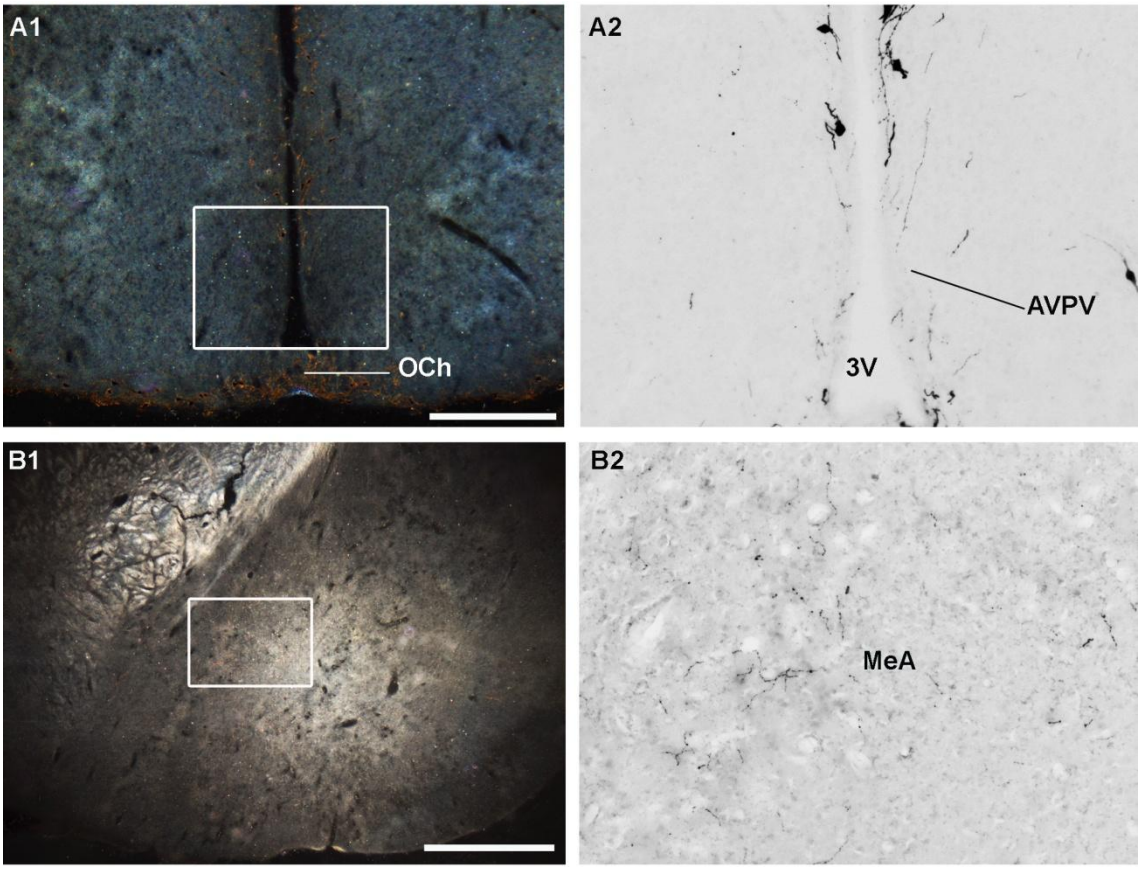


Plate 6.4: Darkfield (A1, B1) and brightfield (A2 and B2) photomicrographs of representative coronal section showing oxytocin-neurophysin-immunoreactive processes within the anteroventral periventricular nucleus and medial amygdala of a female subordinate Damaraland mole-rat. Areas enclosed in a box (A1, B1) are shown in higher magnification (A2 and B2). 3V, Third ventricle; AVPV, anteroventral periventricular nucleus; OCh, optic chiasm; MeA, medial amygdala. Scale bars= 500µm for A1; 1mm for B1.

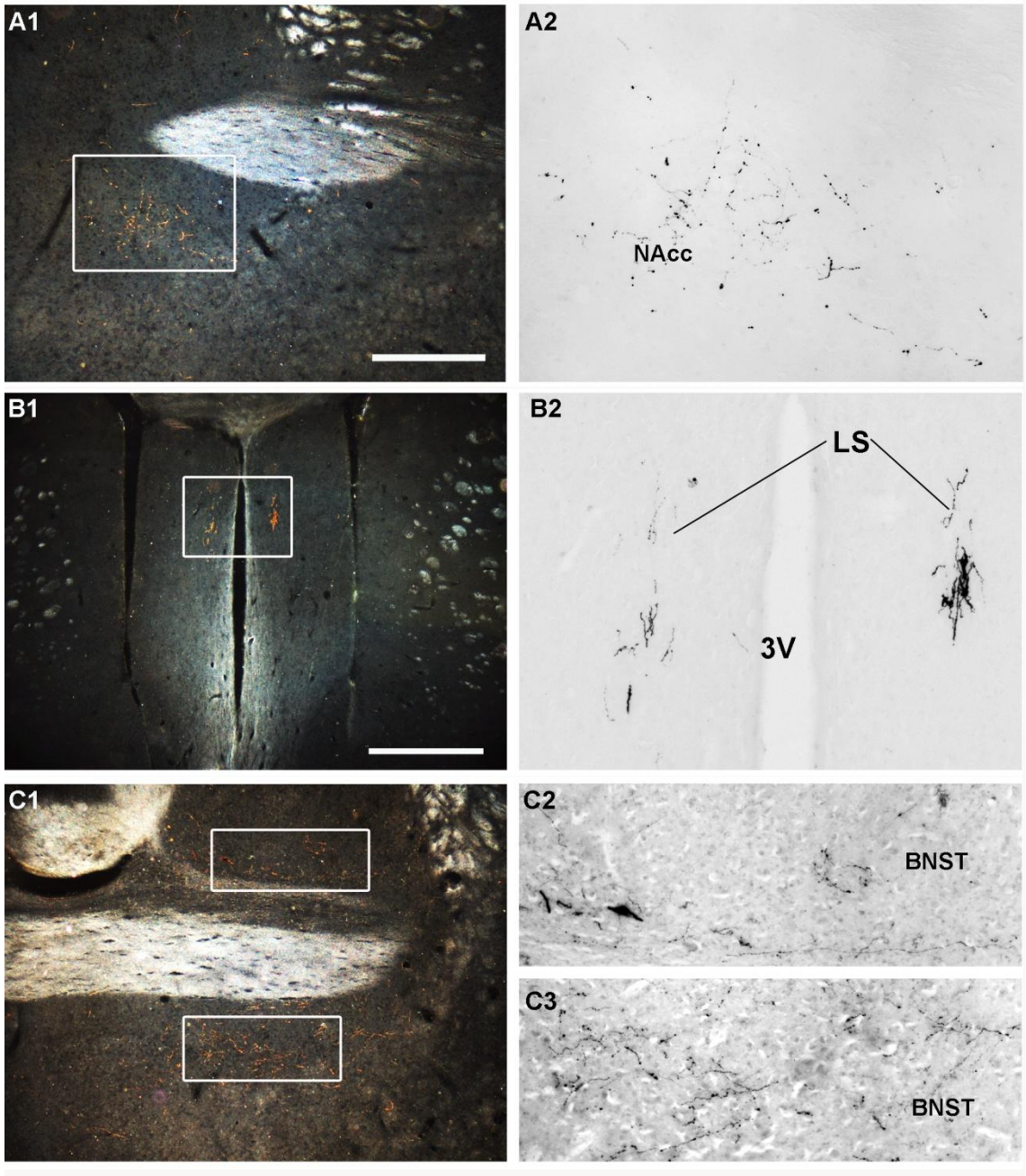


Plate 6.5: Darkfield (A1, B1 and C1) and brightfield (A2, B2, C2 and C3) photomicrographs of representative coronal sections showing oxytocin-neurophysin-immunoreactive processes within the forebrain of a female subordinate Damaraland mole-rat. Areas enclosed in a box (A1, B1 and C1) are shown in higher magnification (A2, B2, C2 and C3). 3V, third ventricle; BNST, bed nucleus of the stria terminalis; LS, lateral septum; NAcc, nucleus accumbens. Scale bars= 500µm for A1 and C1; 1mm for B1.

Distribution of AVP-ir cell bodies

The overall distribution of AVP-ir cell bodies in Damaraland mole-rats was similar to that of other rodents (DeVries *et al.*, 1985; Dubois-Dauphin *et al.*, 1989a; Ferris *et al.*, 1992; Wang *et al.*, 1996; Rosen *et al.*, 2007). The majority of AVP-ir cell bodies were located in the PVH and SON. These AVP-ir cell bodies were darkly stained and spread throughout the PVH. Most of the AVP-ir cell bodies were located on the lateral margins of the PVH, with few located near the lining of the 3V. Scattered AVP-ir cell bodies were distributed ventral to the base of the PVH. No AVP-ir cell bodies were located in the rostral PVH. Within the SON, dense clusters of AVP-ir cell bodies were distributed medio-laterally, extending into the vestigial opt. A few scattered AVP-ir cell bodies were located in the lateral hypothalamus.

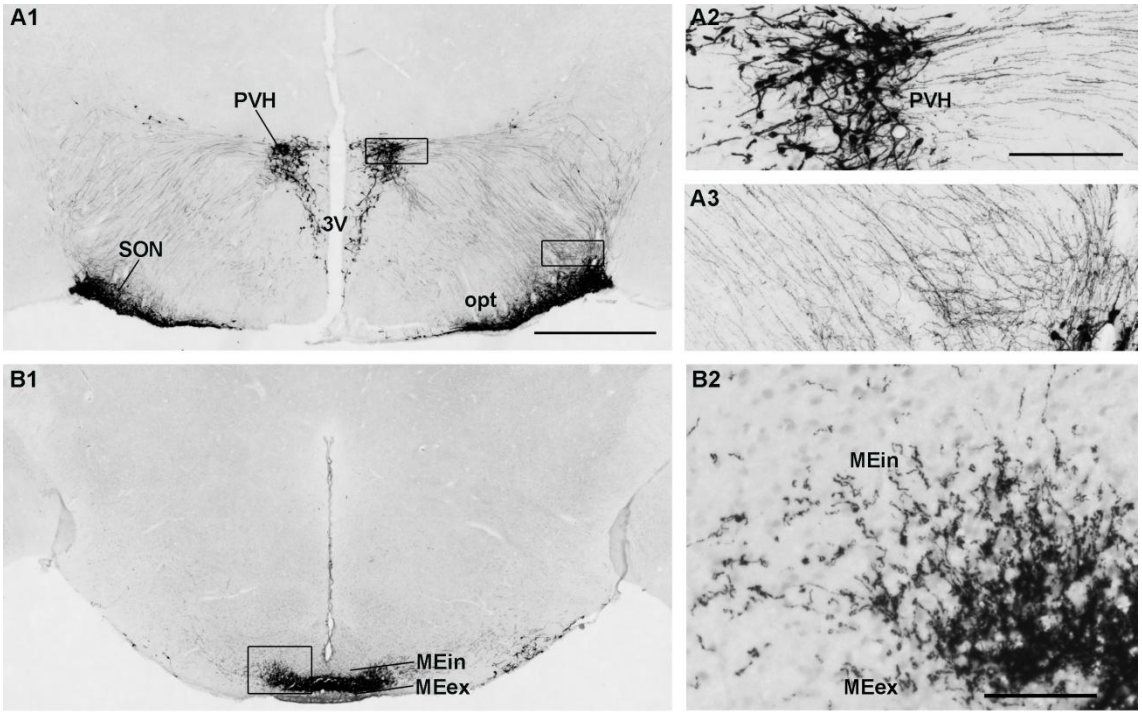


Plate 6.6: Brightfield photomicrographs of representative coronal sections showing: (A1-A3) vasopressin-immunoreactive cell bodies within the paraventricular hypothalamic nucleus and supraoptic nucleus, and (B1-B2) vasopressin-immunoreactive processes within the median eminence of a female subordinate Damaraland mole-rat. Areas enclosed by a box (A1, B2) are shown in higher magnification (A2-A3, B2). 3V, third ventricle; PVH, paraventricular hypothalamic nucleus; MEex, external zone of the median eminence; MEin, internal zone of the median eminence and SON, supraoptic nucleus. Scale bars= 100µm for A1 and B1; 20µm for A2 and A3; 10µm for B2.

Distribution of hypothalamic AVP-ir processes

Within the hypothalamus, the vast majority of processes from AVP-ir cell bodies in the PVH and SON project towards and through the internal zone of the ME (Plate 6.6A1-A3). There were very few AVP-ir processes in the external zone of the ME (Plate 6.6B1-B2). No AVP-ir processes projected towards the 3V (Plate 6.6A1). There were also some scattered AVP-ir processes in the lateral hypothalamus. No AVP-ir processes were observed in the SCN.

Distribution of extra-hypothalamic AVP-ir processes

AVP-ir processes were also distributed in many extra-hypothalamic areas. There were very few (almost negligible) AVP-ir processes present in the MeA (Plate 6.7). In the forebrain, there were virtually no AVP-ir processes in the MS or VDB (Plate 6.8B1-C2). Diffuse networks of AVP-ir processes were located in the NAcc (Plate 6.8A1-A2) and BNST (Plate 6.8D1-D2).

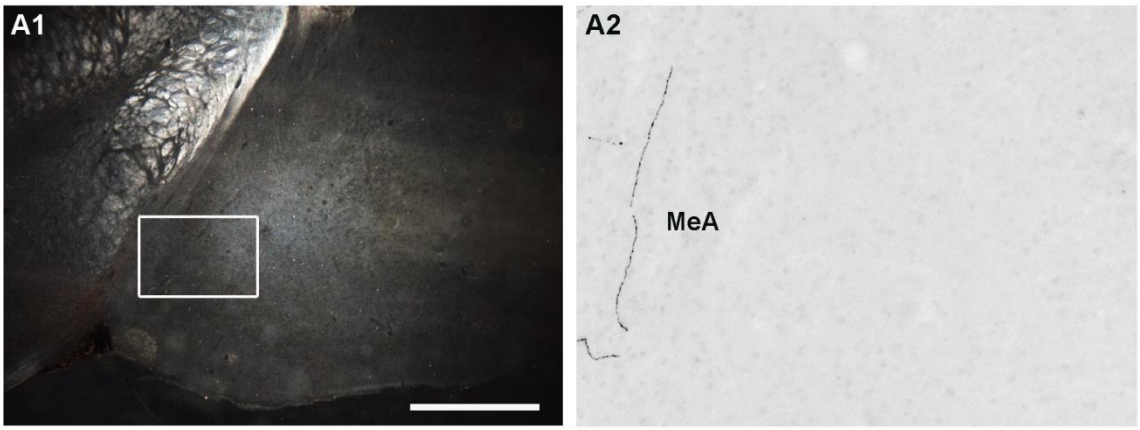


Plate 6.7: Darkfield (A1) and brightfield (A2) photomicrographs of representative coronal sections showing a paucity of vasopressin-immunoreactive processes within the medial amygdala of a female subordinate Damaraland mole-rat. Area enclosed in the box (A1) is shown in higher magnification (A2). MeA, medial amygdala. Scale bar= 1mm for A1.

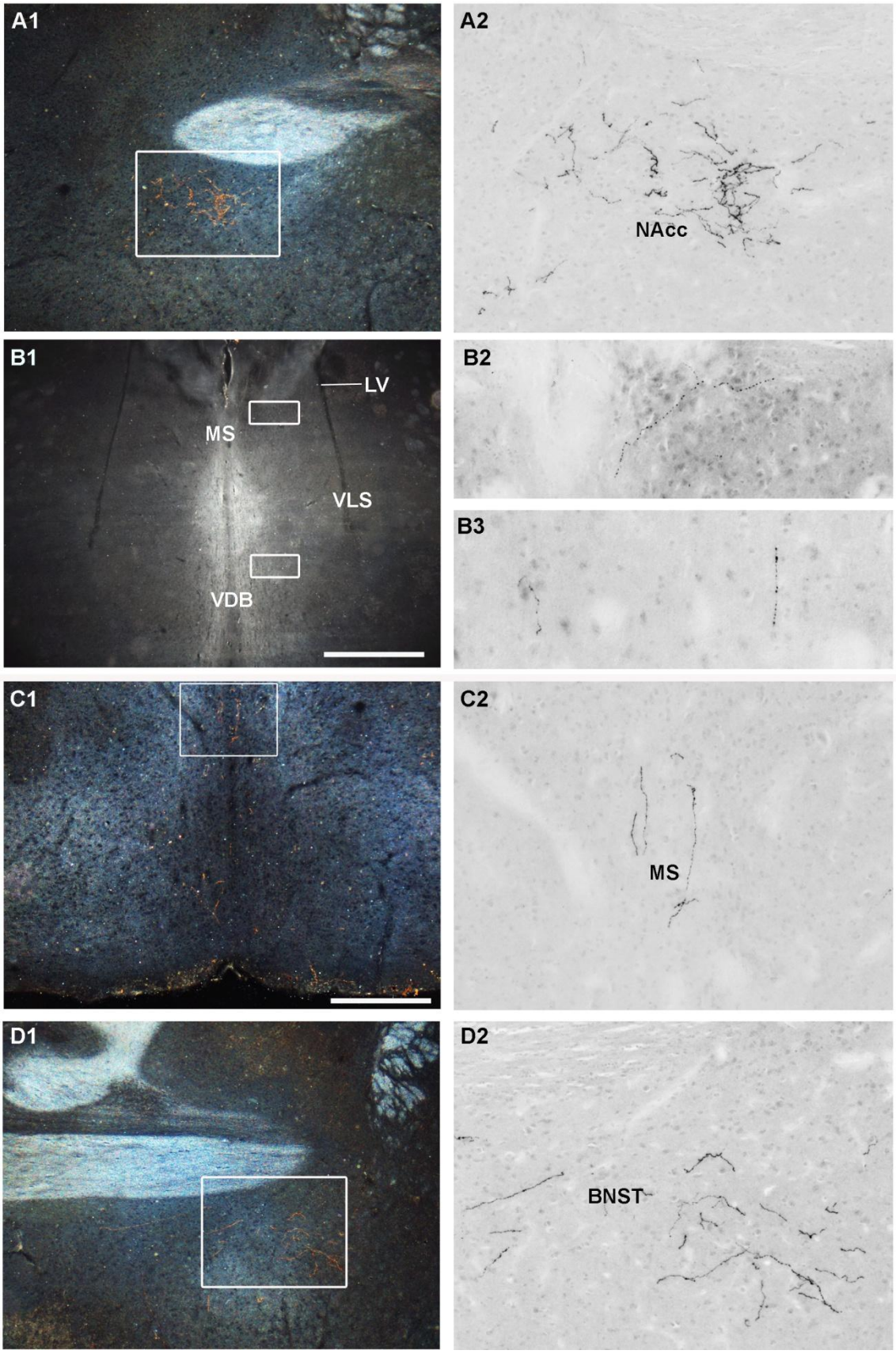


Plate 6.8: Darkfield (A1, B1, C1 and D1) and brightfield (A2, B2, B3, C2 and D2) photomicrographs of representative coronal sections showing vasopressin-immunoreactive processes in the forebrain of a female subordinate Damaraland mole-rat. Areas enclosed in boxes (A1, B1, C1 and D1) are shown in higher magnification (A2, B2, B3, C2 and C3). BNST,

bed nucleus of the stria terminalis; LS, lateral septum; LV, lateral ventricle; MS, medial septum; NAcc, nucleus accumbens, VDB, vertical limb of the diagonal band; VLS, ventrolateral septum. Scale bar= 500µm for C1, A1 and D1; 1mm for B1.

DISCUSSION

Previous research on the diverse range of mammalian social behaviours has implicated the neural OT and AVP systems in mediating the formation and maintenance of social bonds; whether these are between breeding pairs or between parents and offspring (Keverne and Curley, 2004). In this present study, I identified the distribution of OT and AVP and their receptor binding sites in the eusocial Damaraland mole-rat brain.

Oxytocin and its receptor in the nucleus accumbens

As reported in other rodent species for OT, no obvious sex or individual differences were observed in the OT-neurophysin-ir system of Damaraland mole-rats (Wang *et al.*, 1996; Rosen *et al.*, 2008). Previous studies have reported that eusocial naked mole-rats have a dense plexus of OT-neurophysin-ir processes in the NAcc whilst such processes are extremely sparse in the solitary Cape mole-rats (Kalamatianos *et al.*, 2010). Another study also reported that eusocial naked mole-rats have particularly dense innervations of OT-ir processes in the NAcc (Rosen *et al.*, 2008). However, there is only a diffuse network of OT-neurophysin-ir processes in the NAcc of Damaraland mole-rats. Furthermore, a comparable distribution and density of OT-immunoreactivity exists in the NAcc of a wide range of rodents: monogamous prairie voles, promiscuous meadow voles, rats, mice and guinea pigs (Castel and Morris, 1988; Dubois-Dauphin *et al.*, 1989b; Ross *et al.*, 2009a).

In this present study, the highest levels of OTR binding are observed in the shell of the NAcc of eusocial Damaraland mole-rats and in the vicinity of the ICj. In these regions, OT-neurophysin-ir processes are found only in the NAcc. The overall distribution of OT-ir cell bodies in Damaraland mole-rats is very similar to that of other rodents (Buijs *et al.*, 1978; Castel and Morris, 1988; Hermes *et al.*, 1988; Dubois-Dauphin *et al.*, 1989b; Wang *et al.*, 1996; Rosen *et al.*, 2008; Kalamatianos *et al.*, 2010). In a previous study from our laboratory, the results showed that eusocial naked mole-rats exhibit a significantly greater level of OTR binding in the NAcc compared to solitary Cape mole-rats (Kalamatianos *et al.*, 2010). Monogamous prairie and pine voles also have higher OTR densities in the NAcc than the promiscuous meadow and montane voles (Insel and Shapiro, 1992; Olazabal and Young, 2006b). As in the promiscuous voles, OTRs are absent or expressed at low levels in the NAcc of most solitary rodents (Beery *et al.*, 2008; Ross *et al.*, 2009b). Taken together, these studies indicate that all the social mole-rats and voles have high OTR binding in the NAcc whilst all the solitary mole-rats, voles, mice and rats have an absence or low level of OTR binding in the NAcc (Insel and Shapiro, 1992; Olazabal and Young, 2006b; Beery

et al., 2008; Kalamatianos *et al.*, 2010). However, the presence of OTR in the NAcc may not necessarily be a hallmark of social species. In a study comparing OTR binding in social colonial tuco-tuco (*Ctenomys sociabilis*) and the solitary Patagonian tuco-tuco (*Ctenomys haigi*), there was an absence of OTR binding in the NAcc of both species (Beery *et al.*, 2008).

The high level of OTR binding in the NAcc of monogamous voles is thought to contribute to the parental behaviours and the monogamous social structure in these animals (Insel and Shapiro, 1992; Liu and Wang, 2003). For example, blockage of OTRs in the NAcc prevents pair bond formation in female prairie voles (Liu and Wang, 2003). Pharmacological studies indicate OT acts in concert with dopamine in the NAcc to facilitate the formation of monogamous pair bonds (Liu and Wang, 2003). Furthermore, investigation into the NAcc OT system determined that female prairie voles with elevated levels of OTR in the NAcc display accelerated partner preference formation compared with females with lower levels of OTR density (Ross *et al.*, 2009b). However, partner preference is not facilitated in promiscuous meadow voles by introducing oxytocin receptor into the NAcc. These data confirm a role for OTR in the NAcc in the regulation of partner preferences in female prairie voles, and suggest that OTR expression in the NAcc alone is not sufficient to promote partner preferences in solitary species (Ross *et al.*, 2009b). Interestingly, OT and the neurotransmitter dopamine also interact in the NAcc to regulate pair bond formation in female prairie voles. Furthermore, partner preference bonding in female prairie voles is blocked after administration of a dopamine D2 receptor antagonist into the NAcc (Liu and Wang, 2003).

These present findings suggest that oxytocinergic signalling within the NAcc may contribute not only to the monogamous pair bond formation exemplified by the prairie and pine voles, but also exemplified by the sociality between subordinate Damaraland and naked mole-rats in a colony. Although eusocial Damaraland and naked mole-rats are not necessarily monogamous (the queen can mate with up to three males) (Bennett and Faulkes, 2000b), the breeders can form life-long reproductive bonds with these males. Also, subordinate Damaraland and naked mole-rat members participate in active pup care, work cooperatively with conspecifics and are fiercely xenophobic against intruders (Jarvis, 1981; Bennett and Jarvis, 1988; Bennett, 1990; Lacey and Sherman, 1991; Burland *et al.*, 2004). Both the Damaraland mole-rats used in this study and the naked mole-rats investigated in a previous study (Kalamatianos *et al.*, 2010) were subordinate in social status. Therefore, I propose that the NAcc OT system in subordinate animals may instead relate to the fact that many members of the colony actively engage in pup care and other aspects of parental care. For example, a blockade of OTRs in the NAcc of female prairie voles inhibits

spontaneous maternal behaviour (Cho *et al.*, 1999). Apart from mediating affiliative behaviour amongst conspecifics, the NAcc is also implicated in processing pup-related stimuli and regulating alloparental behaviour. Studies on prairie voles suggest that OTRs in the NAcc facilitate allomaternal behaviour by sexually naïve adults or juveniles, moreover, the degree of the allomaternal behaviour can be correlated with OTR density in the NAcc (Olazabal and Young, 2006a,b). Also, allomaternal behaviour can be blocked by the administration of an OTR antagonist into the NAcc (Olazabal and Young, 2006a). Thus, the presence of abundant OTR binding in the NAcc of subordinate Damaraland and naked mole-rats may contribute to the alloparental behaviour practised by these eusocial animals.

Subordinate Damaraland mole-rats fall into two categories (frequent and infrequent workers), where the former are smaller and look after the young in the colony, and the latter are larger and may act as dispersers who build up their own body reserves in preparation for dispersal when environmental conditions are suitable (Scantlebury *et al.*, 2006). Given that the frequent workers are more active participants of pup care, it would be interesting to compare whether there are differences in the OT and OTR NAcc systems between the smaller frequent workers and the larger infrequent subordinate workers.

Oxytocin and its receptor in the islands of Calleja

A striking feature of OTR binding in Damaraland mole-rats is its intensity in the ICj, a similar high density of binding is observed in eusocial naked mole-rats and solitary Cape mole-rats (Kalamatianos *et al.*, 2010). There are disparities between distribution of OT peptide and the distribution of OTR binding in Damaraland mole-rats in this region. In Damaraland mole-rats, there is a high degree of OTR binding in ICj, even though there are no OT-ir processes in the ICj. This same binding/peptide discrepancy has been reported for oxytocin-immunoreactivity in other rodents (Castel and Morris, 1988; Hermes *et al.*, 1988; Dubois-Dauphin *et al.*, 1989b; Kalamatianos *et al.*, 2010).

Oxytocin and its receptor in the septum

There is a distinct lack of OTR binding in the septal regions of the Damaraland mole-rats. However, there is a cluster of OT-neurophysin-ir processes in the LS of Damaraland mole-rats. This discrepancy between binding and peptide distribution is also present in the septum of the eusocial naked mole-rat and the solitary Cape mole-rat. Both naked and Cape mole-rats have dense clusters of OT-neurophysin-ir processes in the LS, with a distinct absence of septal OTR binding (Kalamatianos *et al.*, 2010). Recent studies have identified a negative correlation between the density of OTR binding in the LS and the degree of allomaternal behaviour displayed either within

or between species of voles (Olazabal and Young, 2006b). Promiscuous voles have been shown to have higher levels of OTR binding in the LS than monogamous voles (Insel and Shapiro, 1992; Young *et al.*, 1996; Smeltzer *et al.*, 2006). Similarly, there is a greater level of OTR binding in the LS of solitary tuco-tucos compared with social tuco-tucos (Beery *et al.*, 2008). Although it has been speculated that low LS OTR binding in social species may be permissive for group living, the absence of OTR binding in the LS in both eusocial and solitary mole-rats suggests that the oxytocin-neurophysin-ir processes found in the LS of mole-rats may be vestigial (Kalamatianos *et al.*, 2010). The present findings indicate that differences in behaviour between eusocial mole-rats and solitary Cape mole-rats cannot be attributed to differences in OTR binding in the LS. The roles of oxytocin-dependent actions at this site remain unclear and appears to be species specific.

Oxytocin and its receptor in the extended amygdala

The results of this study report an absence of OTR binding in the MeA and an absence of OT-neurophysin-immunoreactivity in the MeA of Damaraland mole-rats. Comparative studies on social and solitary tuco-tucos suggest that oxytocinergic signalling in the MeA is conducive to social tolerance within group-living rodents (Beery *et al.*, 2008). In mice and rats, this nucleus is implicated in maternal protectiveness (Bosch *et al.*, 2005). Furthermore, there is an elevated level of OTR binding in this nucleus in female rats that are highly responsive to pups (Francis *et al.*, 2000; Champagne *et al.*, 2001; Francis *et al.*, 2002). Eusocial naked mole-rats have a higher density of binding in the MeA than solitary Cape mole-rats, indicating that the absence of OTR binding in the MeA of Damaraland mole-rats may species specific rather than unique to mole-rats (Kalamatianos *et al.*, 2010). Such findings are in accordance with the greater level of OTR binding observed at this site in naked mole-rats, which are highly alloparental, compared with Cape mole-rats, which show minimal maternal behaviour (Kalamatianos *et al.*, 2010). In contrast to this theory, Damaraland mole-rats are also highly alloparental, yet show an absence of OTR binding in the amygdala.

Oxytocinergic signalling in the MeA is crucial for social recognition in mice (Ferguson *et al.*, 2001). In rats, the MeA sends projections to the BNST (Febo *et al.*, 2005). Within the BNST, Damaraland mole-rats display virtually no binding. Naked mole-rats and Cape mole-rats both display OTR binding in the medial and lateral divisions of the BNST (Kalamatianos *et al.*, 2010). In the female rat, the post-parturient period of intense maternal care coincides with increased OTR binding in the lateral BNST (Insel, 1990). In virgin prairie voles, a high level of OTR binding in the lateral BNST has been identified in association with a high incidence of allomaternal behaviour (Insel and Shapiro, 1992). In contrast, polygamous montane voles display

only a low level of OTR binding in the lateral BNST (Insel and Shapiro, 1992). Comparisons of OTR binding between a polygamous and monogamous species within the genus *Peromyscus* identified relatively low binding signals in the lateral BNST of both species (Insel *et al.*, 1991). Thus, OTR binding in the BNST is implicated in parental and alloparental behaviour in certain species (rats and monogamous voles), yet its absence in the BNST of Damaraland mole-rats indicates BNST OTR binding is not an absolute necessity for alloparental behaviour.

Vasopressin and its receptor in the nucleus accumbens

In eusocial Damaraland mole-rats, V1aR binding is most intense in the shell of the NAcc and. AVP-ir processes are also found in the shell of the NAcc. There is no no V1aR binding in the NAcc of eusocial naked mole-rats or solitary Cape mole-rats (T. Kalamatianos and C. W. Coen, unpublished data). V1aR binding has also been observed in the NAcc of the cooperatively-breeding common marmoset monkeys (Wang *et al.*, 1997a).

Vasopressin and its receptor in the septum

A low level of V1aR binding is present in the intermediate and ventral subdivisions of the LS of Damaraland mole-rats. Septal V1a binding is found in solitary Cape mole-rats, eusocial Damaraland and naked mole-rats, and monogamous prairie voles (Kalamatianos and Coen, unpublished; Insel *et al.*, 1994; Lim *et al.*, 2004a). In a previous study comparing V1aR binding in eusocial and solitary mole-rats, there is high levels of V1aR binding in the LS of both eusocial naked and solitary Cape mole-rats, though V1aR binding in the former was more intense (T. Kalamatianos and C. W. Coen, unpublished data). So despite extreme differences in sociality, both naked and Cape mole-rats had intense V1aR binding in the LS. A higher density of V1aR binding is present in the LS of montane voles and this pattern differs to that of monogamous voles species (low V1aR binding in the LS) (Insel *et al.*, 1994; Lim *et al.*, 2004b). In comparison to the distribution of OT-neurophysin-ir processes, there is an absence of AVP-immunoreactivity in the LS of Damaraland mole-rats. There was a dense cluster of AVP-ir processes in the LS of eusocial naked mole-rats and a negligible amount of AVP-immunoreactivity in the LS of solitary Cape mole-rats (T. Kalamatianos and C. W. Coen, unpublished data).

The Damaraland mole-rats used in this study and the naked mole-rats from a previous study (Kalamatianos *et al.*, 2010) were subordinates who had low levels of circulating gonadal hormones. The AVP system has also been shown to be both sexually differentiated and regulated by gonadal hormones in rodents. In rats, sex differences in the AVP-immunoreactivity and expression persist following

gonadectomy, indicating that sex differences in AVP mRNA expression are organised early in development (Wang, 1994). Another unusual aspect of the AVP system in Damaraland and naked mole-rats is the lack of sex differences in the distribution of AVP-immunoreactivity (Rosen *et al.*, 2007). Sex differences in AVP-immunoreactivity in the LS are widespread amongst vertebrates. In fact, the AVP-innervation of the LS (by neurones from the BNST and MeA) is one of the most consistently found sexually differentiated brain regions; with males having more cells and denser projections to the LS than females (De Vries and Panzica, 2006). The absence of sexual differentiation in AVP-innervations in the LS of Damaraland and naked mole-rats is perhaps not so surprising given that brain regions that are sexually dimorphic in most typical rodents are sexually monomorphic in eusocial Damaraland and naked mole-rats (Anyan *et al.*, 2011; Holmes *et al.*, 2011). Rather, neural morphology in these eusocial mole-rats appears to be related to breeding status rather than sex (Peroulakis *et al.*, 2002; Seney *et al.*, 2006; Holmes *et al.*, 2011).

Whilst the AVP projections to the pituitary (which arise from the PVH and SON) are evolutionarily conserved, the extrahypothalamic AVP circuits (especially in the VP, BNST and LS) are species specific and change rapidly over evolutionary time (Goodson and Bass, 2001). Previous studies on monogamous and promiscuous voles have reported both sex and species differences in AVP-immunoreactivity. Monogamous male prairie voles also had a higher density of AVP-ir processes in the LS than promiscuous male meadow voles (Wang, 1995). But overall, species differences in AVP-immunoreactivity were minimal compared to sex differences. Males of both species had significantly higher density of AVP-ir processes in the LS and VP than females (Wang, 1995; Wang *et al.*, 1996; Lim *et al.*, 2004a). These sexually dimorphic AVP pathways in the LS in voles closely resemble those in other species of rodents, like rats, mice, hamsters and gerbils (van Leeuwen *et al.*, 1985; Buijs *et al.*, 1986; Hermes *et al.*, 1990; Crenshaw *et al.*, 1992). These immunohistochemical studies indicate that although subtle species differences were found, the distribution patterns of AVP-ir neurones between monogamous and promiscuous vole species are highly conserved. Like Damaraland and naked mole-rats, female voles also had virtually no AVP-ir processes in the LS, regardless of species.

Interestingly, the exact distribution of V1aR binding is unique in all species examined to date, and the differences have been linked to the divergent patterns of social behaviour. Similarly, large species differences are also observed in the presence or absence of AVP-immunoreactivity in the LS, as mentioned above. Despite sharing a similar social structure, social species (e.g. monogamous voles and eusocial mole-rats) differ in more respects than just their mating system and biparental care. Hence, the differences in the AVP system between species may also be associated with another

aspect of behaviour, such as aggression (Bester-Meredith *et al.*, 1999). For example, relative to more asocial territorial species, gregarious songbirds show greater induction of Fos within AVT (avian version of AVP) neurones after a social stimulus, a greater number of AVT neurones in the BNST and a higher density of V1aR-like binding sites in the LS (Goodson and Bass, 2001).

Other studies have suggested that V1aR binding within the LS may be associated with social spacing rather than with mating systems (which may be correlated with V1aR distribution in other brain areas, like the VP) (Goodson and Bass, 2001). The monogamous voles (low V1aR binding in LS) occupy a large home range with a large degree of overlap with other adult conspecifics, whereas the promiscuous voles (high V1aR binding in LS) occupy much more exclusive home ranges (Hofmann *et al.*, 1984; Tamarin, 1985; Wolff, 1985a). However, in *Peromyscus californicus*, the monogamous mice (high V1aR binding in LS) occupy exclusive home range whilst the promiscuous mice (low V1aR binding in LS) occupy large home ranges that overlap with other conspecifics (Wolff, 1985b; Ribble and Salvioni, 1990). For species in the *Microtus* and *Peromyscus* family, the species which occupies a more exclusive home range exhibits a higher density of V1aR binding in the LS (Insel *et al.*, 1991; Bamshad *et al.*, 1993; Insel *et al.*, 1994; Young *et al.*, 1997; Bester-Meredith *et al.*, 1999). This hypothesis is particularly intriguing because arginine vasotocin, an AVP homologue, modulates territorial behaviour in birds (Goodson and Bass, 2001), and V1aR expression in several brain regions is correlated with communal living in female tuco-tucos (Beery *et al.*, 2008) and with social spacing in singing mice (Campbell *et al.*, 2009a).Thus, these observations indicate that species differences in V1aR binding in the LS may reflect spacing behaviour rather than whether a species displays monogamous or promiscuous behaviour.

Furthermore, it has been argued that aggression is facilitated in those species with a high overlap in home territories, such as prairie voles (Goodson and Bass, 2001). Solitary Cape mole-rats have an exclusive home range (mean burrow length=48 metres) and a high density of V1aR binding in the LS (Jarvis and Bennett, 1991; Kalamatianos *et al.*, 2010). However, both the naked and Damaraland mole-rats occupy large home ranges (mean burrow length=595-3027 metres) (Jarvis and Bennett, 1991). Although, V1aR binding in the LS of Damaraland mole-rats is low, the level of V1aR binding in the LS of naked mole-rats is very high and this finding goes against the spacing hypothesis. Whether this spacing hypothesis is an accurate predictor correlating convergence in behaviour and neuroanatomy in rodents remains to be determined.

Vasopressin and its receptor in the ventral pallidum

There is an absence of V1aR binding in the VP of eusocial Damaraland mole-rats. Intriguingly, there is intense V1aR binding in the VP of solitary Cape mole-rats and no V1aR binding in the VP of eusocial naked mole-rats (T. Kalamatianos and C. W. Coen, unpublished data). Interestingly, previous studies have also found extreme differences in V1aR binding in different vole species. The differences in the social behaviour of monogamous and promiscuous voles can be correlated with the variation in the distribution patterns and regional densities of V1aRs. Monogamous prairie and pine voles showed a similar pattern of V1aR binding (high V1aR binding in the VP) and this pattern differed to that of promiscuous vole species (low V1aR binding in the VP) (Insel *et al.*, 1994; Wang *et al.*, 1997b; Young *et al.*, 1997; Lim *et al.*, 2004b; Smeltzer *et al.*, 2006). These remarkable species differences in V1aR binding are thought to relate to the different life strategies of monogamous and promiscuous voles (Insel *et al.*, 1994; Young *et al.*, 1997).

Moreover, it is thought that these differences in V1aR binding and life strategies may be associated with the species differences in the promoter regions of the V1aR gene. Monogamous prairie and pine voles have several repetitive microsatellite DNA sequences in the promoter region of the V1aR gene that are not present in the promiscuous meadow or montane voles (Young, 1999; Young *et al.*, 1999a; Hammock and Young, 2002,2004). Additionally, solitary mice carrying a transgene coding for the monogamous prairie vole V1aR had a forebrain V1aR distribution pattern and showed affliative behaviour similar to monogamous prairie voles (Young *et al.*, 1999a). The overexpression of V1aR through a viral vector into the forebrain of promiscuous male meadow voles increases pair-bond formation to the level seen in monogamous prairie voles, even without mating (Lim *et al.*, 2004b). Despite this increase in partner preference, over expression of V1aR does not increase paternal care in the promiscuous male meadow voles. This suggests that the neural circuits underlying paternal care and pair-bond formation are distinct. Indeed, infusions of V1aR antagonists into the forebrain selectively blocks female partner preference but not paternal care in male prairie voles, whereas infusions into the MeA blocks paternal care, but not partner preference in male prairie (Lim *et al.*, 2004b).

Interestingly, the forebrain expression of V1aR is also higher in monogamous California deer mice (*Peromyscus* genus) and cooperatively-breeding marmoset monkeys when compared with that of other closely related promiscuous species, indicating the AVP-mediated circuits have convergently evolved to mediate affiliative behaviour in monogamous species (Bester-Meredith *et al.*, 1999; Young, 1999; Young *et al.*, 1999b). In a recent study investigating the vasopressin system in eight different species of deer mice (genus *Peromyscus*) with varying social structures, the authors

find a lack of clear, consistent relationship between V1aR expression pattern and social behaviour in *Peromyscus (*Turner *et al.*, 2010). Moreover, there is no correlation between the mating system of *Peromyscus* species and repeat length at any of three identified V*1aR* microsatellite loci (Turner *et al.*, 2010). This suggests that the precise molecular mechanism causing divergence in mating behaviour is not the same as that proposed for *Microtus* voles. The authors found neither the pattern reported in *Microtus* (higher expression in monogamous species in the VP and lower expression in the LS) nor any other correlation between expression and mating system (Turner *et al.*, 2010). Hence, it is unlikely that V1aR plays a convergent role in regulating mating systems. Also, the results presented here do not support an association between V1aR expression and social spacing in *Peromyscus* species. Overall, these results indicate that the genetic determinants of monogamy in mammals may be more complex than a simple molecular switch that turns on pair-bonding behaviour, as reported in some vole species.

Vasopressin and its receptor in the hypothalamus

The overall distribution of AVP-ir cell bodies in Damaraland mole-rats is similar to that of other rodents (Caffe and van Leeuwen, 1983; DeVries *et al.*, 1985; Whitnall *et al.*, 1985; Castel and Morris, 1988; Dubois-Dauphin *et al.*, 1989a; Albers *et al.*, 1991; Bamshad *et al.*, 1993; Rosen *et al.*, 2007) and similar to the distribution of OT-ir cell bodies in Damaraland mole-rats. Damaraland mole-rats have a typical rodent neurohypophysial AVP system with AVP-ir cell bodies in the PVH and SON, and AVP-ir processes projecting from these nuclei to the ME. Scattered AVP-ir cell bodies are also distributed in the lateral hypothalamus. There are disparities between distribution of AVP peptide and the distribution of V1aR binding in Damaraland mole-rats. For example, there was a high level of V1aR binding the AVPV of Damaraland mole-rats, but there was an absence of the peptide. Intriguingly, there is a high level of V1aR binding in the NAcc and AVPV of Damaraland mole-rats. Although the AVPV has not been implicated as a V1aR binding site in other rodents, the AVPV is the primary site of kisspeptin neurones which are necessary for puberty-onset and fertility maintenance in mammals (Clarkson *et al.*, 2009b). However, the Damaraland mole-rats used in this study were subordinate and (in chapter 4 of this thesis) subordinate Damaraland mole-rats have virtually no RFamide-ir cell bodies in the AVPV. Also, in a previous studies on naked and Cape mole-rats, no evidence of V1aR binding in the AVPV was observed in either of these mole-rats species (Kalamatianos *et al.*, 2010). This suggests that V1aR binding in the AVPV is specific to Damaraland mole-rats and no other mole-rat species so far studied.

In most mammals studied to date, AVP-ir cell bodies have been found in the SCN (Ben-Barak *et al.*, 1985; Albers *et al.*, 1991; Bamshad *et al.*, 1993; Wang *et al.*, 1996). Similar to the OT-immunoreactivity findings reported above, there are no AVP-ir cell bodies or processes in the SCN of Damaraland or naked mole-rats, regardless of sex (Rosen *et al.*, 2007). It is thought that the absence of these cells in the SCN may relate to their subterranean environment where light exposure is negligible. Interestingly, other subterranean rodents, such as the blind mole-rat (*Spalax ehrenbergi*) also display a paucity of AVP-ir cell bodies in the SCN (Negroni *et al.*, 1997).

Although in naked mole-rats, no evidence of sexual differentiation or breeding status differences were found in the number of AVP-ir cells in the BNST or LS, the number of AVP-ir cell bodies in the DMH is greater in breeders than subordinates of both sexes; and this difference was especially greater between breeding and subordinate females (Rosen *et al.*, 2007). This is interesting since the DMH in mammals has been linked to social behaviours, such as sexual and maternal behaviours and lactation (Sharma and Rissman, 1994; Chen and Smith, 2003; Mann and Babb, 2004). In naked mole-rat colonies, only the one dominant queen breeds and is able to meet the energetic demands lactation (Jarvis, 1991; Lacey and Sherman, 1991). Like in naked mole-rats, no sexual differentiation of the AVP system was observed in females spotted hyenas (*Crocuta crocuta*). Interestingly, female spotted hyenas are also relatively sexually monomorphic, like naked and Damaraland mole-rats, and have a highly masculinised external genitalia (Rosen *et al.*, 2006; Rosen *et al.*, 2007). Again, like the sexually monomorphic naked mole-rats, there were no sex differences in AVP-immunoreactivity between female and male spotted hyenas (Rosen *et al.*, 2006).

Evolutionary and global perspectives

This study has identified marked differences in the distribution of OT- and AVP-immunoreactivity, and the telencephalic OTR and V1aR binding sites in eusocial Damaraland mole-rats. Damaraland mole-rats have species specific distribution of OTR and V1aR binding in the telencephalon with some unusual characteristics in the forebrain (see Table 6.1 and figure 6.1 for a summary). One important question is whether evolutionary convergence in particular behavioural characteristics can be reliably correlated with convergence in peptide mechanisms. It is important to note that our study was not designed to elucidate causal relationships between species differences in OTR/V1aR binding and the species differences in social behaviour. Rather, the functional significance of the species specific differences in OTR and V1aR binding remains to be determined.

Our conclusions are:

1) Oxytocin and its receptor in the NAcc:
    a) Both are present in the NAcc of eusocial mole-rats
    b) Both are absent in the NAcc of solitary mole-rats
    c) Both are present in the NAcc of monogamous voles
    d) OTR is absent in the NAcc of promiscuous voles, although OT is present.

Firstly, I speculate that the abundance of OTR binding and its peptide in the NAcc in eusocial mole-rats suggests that they underlie aspects of sociality and cooperation, without necessarily conferring monogamy as in monogamous voles. Secondly, given the origins of eusocial Damaraland mole-rats from ancestors common to solitary Cape mole-rats (rather than eusocial naked mole-rats), the possibility of convergent evolution merits attention. Thirdly, the presence of oxytocin and its receptor in the NAcc in both eusocial mole-rats and monogamous voles may be an example of convergent evolution or genetic conservation between old world eusocial mole-rats and new world monogamous voles.

2) Vasopressin & its V1aR in the VP:
    a) Both are absent in the VP of eusocial mole-rats
    b) V1aR is present in the VP of solitary mole-rats, but AVP is absent
    c) V1aR is present in the VP of monogamous voles, but AVP is present in males only
    d) V1aR is absent in the VP of promiscuous voles, although AVP is present in males

Given that AVP and V1aR in the VP contribute to pair-bonding in male voles, the presence of V1aR at that site without an apparent ligand in solitary Cape mole-rats raises the possibility of a lost ancestral function.

3) Vasopressin & its V1aR in the NAcc:
    a) Both are present in eusocial Damaraland mole-rats.
    b) Both are absent in eusocial Naked mole-rats.
    c) Both are ansent in solitary Cape mole-rats.
    d) Both are absent in monogamous & promiscuous voles.

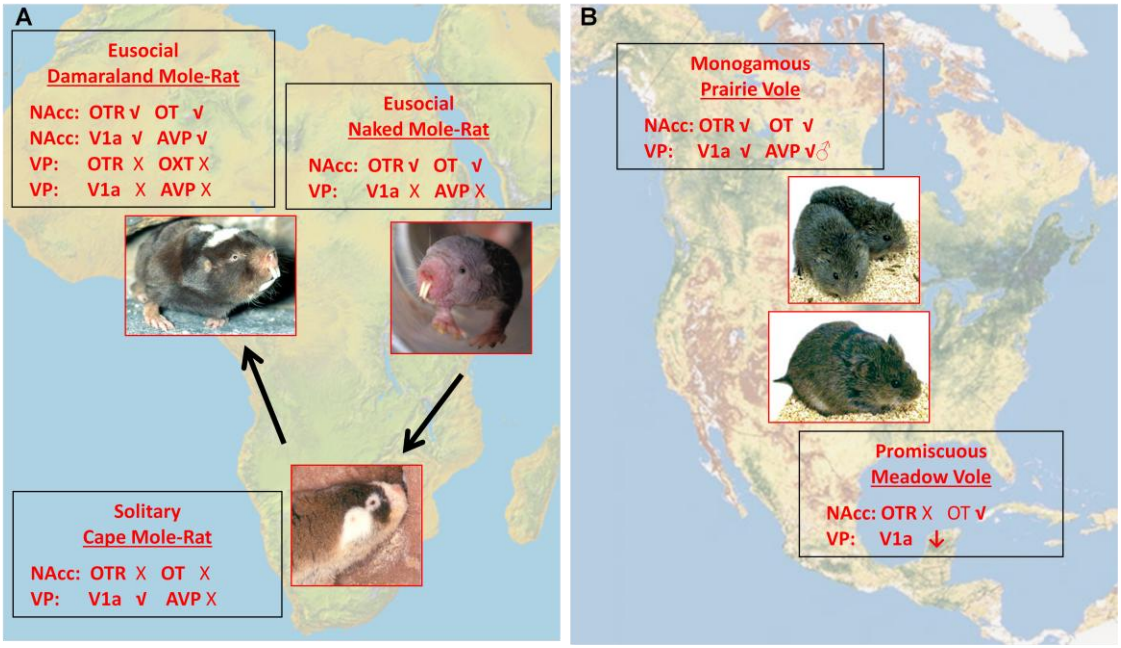
Given the evidence that eusociality has evolved twice in the family Bathyergidae, it is possible that the origins of Damaraland eusociality relate to parallel emergence of V1aR and OTR and their respective ligands in the NAcc. Secondly, the emergence of V1aR in the AVPV of eusocial Damaraland mole-rats may have evolved independently as these animals diverged from the common ancestor and radiated north into Central Africa.

Table 6.1: Distribution of oxytocin and vasopressin, and their receptors in the brains of three mole-rats species

Species			Naked mole-rat	Cape mole-rat	Damaraland mole-rat
OXT	Nacc	Binding	~~xxx~~	–	~~xx~~
		Peptide	~~xxx~~	–	~~xx~~
	BNST	Binding	~~xx~~	~~x~~	~~x~~
		Peptide	~~xxx~~	~~xxx~~	~~xx~~
	VP	Binding	–	–	–
		Peptide	–	–	–
	LS	Binding	–	–	–
		Peptide	~~xx~~	~~xx~~	~~x~~
AVP	Nacc	Binding	–	–	~~xxx~~
		Peptide	~~xx~~	~~x~~	~~xx~~
	BNST	Binding	–	–	–
		Peptide	~~xx~~	~~x~~	~~xx~~
	VP	Binding	–	~~xxx~~	–
		Peptide	–	–	–
	LS	Binding	~~xxx~~	~~xxx~~	~~xx~~
		Peptide	~~x~~	~~x~~	–

Pink columns indicate eusocial, blue column indicates solitary species. ~~xxx~~= high, ~~xx~~= medium, ~~x~~= low, –= absent

Figure 6.1: Cross-species comparison of oxytocin and vasopressin, and their receptor systems, in (A) Old World mole-rats in Africa and (B) New World voles in North America.



Black arrows in A represent the progress of mole-rats through evolutionary time from a common ancestor. Ticks represent presence and crosses represent absence. ♂= males only.

CHAPTER 7:

Adult neurogenesis in the hippocampal dentate gyrus of reproductively-activated and subordinate naked mole-rats (*Heterocephalus glaber*)

ABSTRACT

Eusocial naked mole-rats live in long and complex burrow systems, and spend most of their time foraging for underground tubers which are spread diffusely. Within a colony structure, spatial learning and memory is an important skill for the survival. This is particularly true for naked mole-rats, animals who explore a subterranean niche that is associated with high travelling costs. Pubertal development is marked by significant decreases in cellular proliferation and neurogenesis in the dentate gyrus (DG) of the hippocampus. It is thought that gonadal steroid hormones modulate neurogenesis during the transition from juvenile to adult status. New neurones in the DG may become incorporated into the neural circuitry of the hippocampus where they may participate in a range of functions, such as, learning and memory. The present study was conducted to determine: (1) the levels of adult hippocampal neurogenesis in naked mole-rats, and (2) whether changes in social status have an effect on adult hippocampal neurogenesis. In this chapter, BrdU-immunoreactivity was analysed in subordinate naked mole-rats to determine levels of adult hippocampal neurogenesis, doublecortin- (DCX) immunoreactivity was analysed in subordinate and reproductively-activated naked mole-rats. The number of BrdU- and DCX-positive cells in the DG was low, indicating that the levels of hippocampal neuronal proliferation are low in naked mole-rats. Furthermore, there was no significant effect of sex or reproductive status. The results of this study indicate that naked mole-rats have a remarkably low rate of adult hippocampal neurogenesis. Previous studies on bats and humans have also discovered a remarkably low rate of neurogenesis in these animals, indicating that a high levels of neurogenesis in the hippocampus is not crucial for a variety of cognitive functions, like spatial reference memory in a complex environment. Two factors linking the low neurogenic capacity of naked mole-rats, bat and humans are longevity and group living. I propose that low hippocampal neurogenesis may be common to long-lived social species.

INTRODUCTION

Within a colony structure, spatial learning and memory is an important skill for the survival and may vary between individuals who have different roles. This is particularly true for animals that explore the subterranean niche as it is associated with high travelling costs. Eusocial naked mole-rats live in long and complex burrow systems, and spend most of their time foraging for underground tubers which are spread diffusely (Jarvis and Bennett, 1991; Bennett and Faulkes, 2000a). The DG of the adult hippocampus produces new neurones which become incorporated into the neural circuitry of the hippocampus. Previous studies have suggested that these hippocampal neurones may participate in a range of hippocampal functions, such as, learning and memory (Barnea and Nottebohm, 1996; Gould et al., 1999; Kempermann, 2002). Thus, adult hippocampal neurogenesis may be a marker for cognitive functions.

Apart from the cognitive challenges of fossorial life, individuals within a naked mole-rat colony vary in social status and perform different roles within the colony. Whilst reproductive behaviour remains exclusive to the breeders, subordinate members of the colony perform supporting roles, such as foraging, care for offspring and colony defence (Faulkes *et al.*, 1990a; Faulkes and Abbott, 1991; Faulkes *et al.*, 1991; Lacey and Sherman, 1991). The reproductive suppression of subordinate female and male naked mole-rats is profound. Subordinates of both sexes have a suppressed HPG axis and can become reproductively-activated when isolated from the queen (Faulkes *et al.*, 1990a; Faulkes *et al.*, 1990b; Faulkes and Abbott, 1991; Faulkes *et al.*, 1991; Jarvis, 1991; Faulkes *et al.*, 1994; Clarke and Faulkes, 1998). Pubertal development is marked by significant decreases in cellular proliferation and neurogenesis in the DG of the hippocampus. It is thought that gonadal steroid hormones modulate neurogenesis during the transition from juvenile to adult status. In a study in male rats, BrdU and DCX cell numbers in the DG were found to decrease during pubertal development along with increases in their plasma testosterone levels, and this pubertal-related decline in neurogenesis was not effect by gonadectomy (Ho *et al.*, 2011). Other studies have reported that subordinate naked mole-rats undergo significant changes in their neuroanatomy when they become reproductive, moreover, these changes are independent of gonadal steroids (Holmes *et al.*, 2009; Holmes *et al.*, 2011).

The present study was conducted to determine: (1) the levels of adult hippocampal neurogenesis in naked mole-rats (fossorial animals living in burrows with complex spatial navigational requirements), and (2) whether changes in social status have an effect on adult hippocampal neurogenesis. In this chapter, BrdU-immunoreactivity was analysed in subordinate naked mole-rats to determine levels of

adult hippocampal neurogenesis, DCX-immunoreactivity was analysed in subordinate and reproductively-activated naked mole-rats.

MATERIALS AND METHODS

Study animals

The BrdU immunohistochemistry experiment compared 2 groups of naked mole-rats: (group 1) female subordinates (N= 4) and (group 2) male subordinates (N= 3). The DCX immunohistochemistry experiment compared 4 groups of naked mole-rats: (group 1) female reproductively-activated non-breeders (N= 3); (group 2) male reproductively-activated non-breeders (N= 3); (group 3) female subordinates (N= 9) and (group 4) male subordinates (N= 6). Only DCX antibody was used to analyse the 4 different groups of naked mole-rats due to the limited number of brain sections available for this study.

BrdU and doublecortin immunohistochemistry

BrdU is an exogenous cell tracer that can be used to visualise neurogenesis. It is a thymidine analog and incorporates into dividing cells during DNA synthesis. In order to immunostain for BrdU, the naked mole-rats studied were given four i.p. injections of 50 mg/kg BrdU (Sigma-Aldrich) 2 hours apart 24 hours before they were sacrificed. BrdU solution was prepared by dissolving BrdU in warm saline solution (0.9% w/v NaCl in sterile H_2O) with the addition of a few drops of NaOH (0.01 M) until a solution of pH7 is achieved, the solution was used immediately. The injection volume of BrdU solution was adjusted in proportion of the weight of the animal.

One series of brain sections were immunostained for BrdU (polyclonal sheep anti-BrdU 1:2000; Sigma-Aldrich) and another series of brain sections were immunostained for DCX (polyclonal goat anti-DCX 1:300; Santa Cruz Biotechnology) as described above in chapter 3 for GnRH-1 immunohistochemistry. For BrdU immunohistochemistry, brain sections were first incubated in 2 M hydrochloric acid for 30 min (in order to denature the DNA to allow access for the BrdU antibody) and the acid was then neutralised by rinsing brain sections in 0.1 M PBS solution. The secondary antibody used was biotinylated donkey anti-sheep IgG (for sheep antibody) or biotinylated donkey anti-goat IgG (for goat antibody) (1:1000; Stratech, Newmarket, Suffolk, UK).

Digital photomicrographs

Brightfield photomicrographs were obtained using a Nikon E600 microscope at magnifications of x40, x100 and x200 with a Micro-Publisher 5.0 camera (InterFocus Imaging, Cambridge, UK). The camera was controlled by MCID Core software (Interfocus Imaging). Images were later post-processed using Adobe Photoshop, CS3 to adjust brightness and contrast. Background artefacts were removed as necessary; no other modifications were made to images. Final images were compiled into multi-panel plates in Microsoft Publisher, minor changes to brightness and contrast were made after importing into Microsoft Publisher if necessary.

Quantification and statistical analysis

BrdU- and DCX-immunoreactivity were analysed in the brains of naked mole-rats. Slides were masked and coded for analysis. For each animal, the numbers of BrdU- and DCX-positive cells in the DG of each hippocampal arm were counted by eye with a Nikon E600 microscope at X200 magnification. BrdU-positive cells in the DG of each hippocampal arm were quantified for anatomically matched hippocampal sections for each animal. A density score for BrdU was not calculated since the number of BrdU-ir cell bodies in the DG was very low, so this was deemed unnecessary. The number of DCX-ir cell bodies in the DG of each hippocampal arm were counted in brain sections between -3.24 mm to -4.56 mm from Bregma (Paxinos and Watson, 2007). A density score for DCX-immunoreactivity per mm was calculated by: (1) counting the number of DCX-ir cell bodies in the entire length of the DG, (2) measuring the length of the SGZ of the DG (in µm) using the MCID Core line measurement tool (DG lengths ranged from 629 µm to 1536 µm), (3) dividing 1 mm by the length of the DG, and finally (4) multiplying this ratio by the number of DCX-ir cell bodies to obtain a density score of the number of DCX-ir cell bodies per mm of DG. For example: if there were 50 DCX-ir cell bodies and the DG measured 1.2 mm, we would multiple 50 by 1/1.2 to obtain a density score of 41.7 DCX-ir cell bodies per mm. A mean density score per mm was then calculated by adding up the DCX density scores in each animal and dividing by the number of individual DG sections analysed, this gave a mean density score per mm for each animal.

For all comparisons, statistical significance was set at $p < 0.05$ An independent samples T-test was undertaken to determine whether there was a significant difference in the number of DG BrdU-positive cells between females and males. A one-way ANOVA test was undertaken to determine whether there was a significant effect of group on the mean density of DCX-ir cell bodies. If the overall one-way ANOVA test was statistically significant, then each animal group was compared with one-another using a post-hoc Tukey test. A Pearson's correlation test was done to determine

whether there was a significant correlation between the number of DG BrdU-positive cells and body mass, and between the mean density of DCX-positive cells per mm and body mass.

RESULTS

The distribution and quantification of BrdU-immunoreactivity

Moderate numbers of BrdU-positive cells were around the OV of the OB (Plate 7.1 A1-A2) and SVZ (Plate 7.1B1-B2). In the SVZ, BrdU-positive cells were present tightly lining the ependymal layer of the SVZ and were hard to distinguish from glial cells (Plate 7.1B1-B2). As a result, the number of BrdU-positive cells were not counted in the SVZ. In the hippocampus, BrdU-positive cells were located in the SGZ of the DG, and there were no BrdU-positive cells present in the hillus of the DG (Plate 7.1C1). The number of BrdU-positive cells in the DG was very low, indicating that the levels of hippocampal neuronal proliferation are low. Female naked mole-rats had a mean number of 1.49 (± 0.509 SEM) BrdU-positive cells per DG section. Male naked mole-rats had a mean number of 2.76 (± 0.277 SEM) BrdU-positive cells per DG section. There was no significant difference in the number of BrdU-positive cells between female and males; $t(5)= 1.969$, $p= 0.106$). Furthermore, there was no significant correlation between number of BrdU-positive cells and body mass ($r= 0.587$, $p= 0.166$) (Figure 7.1).

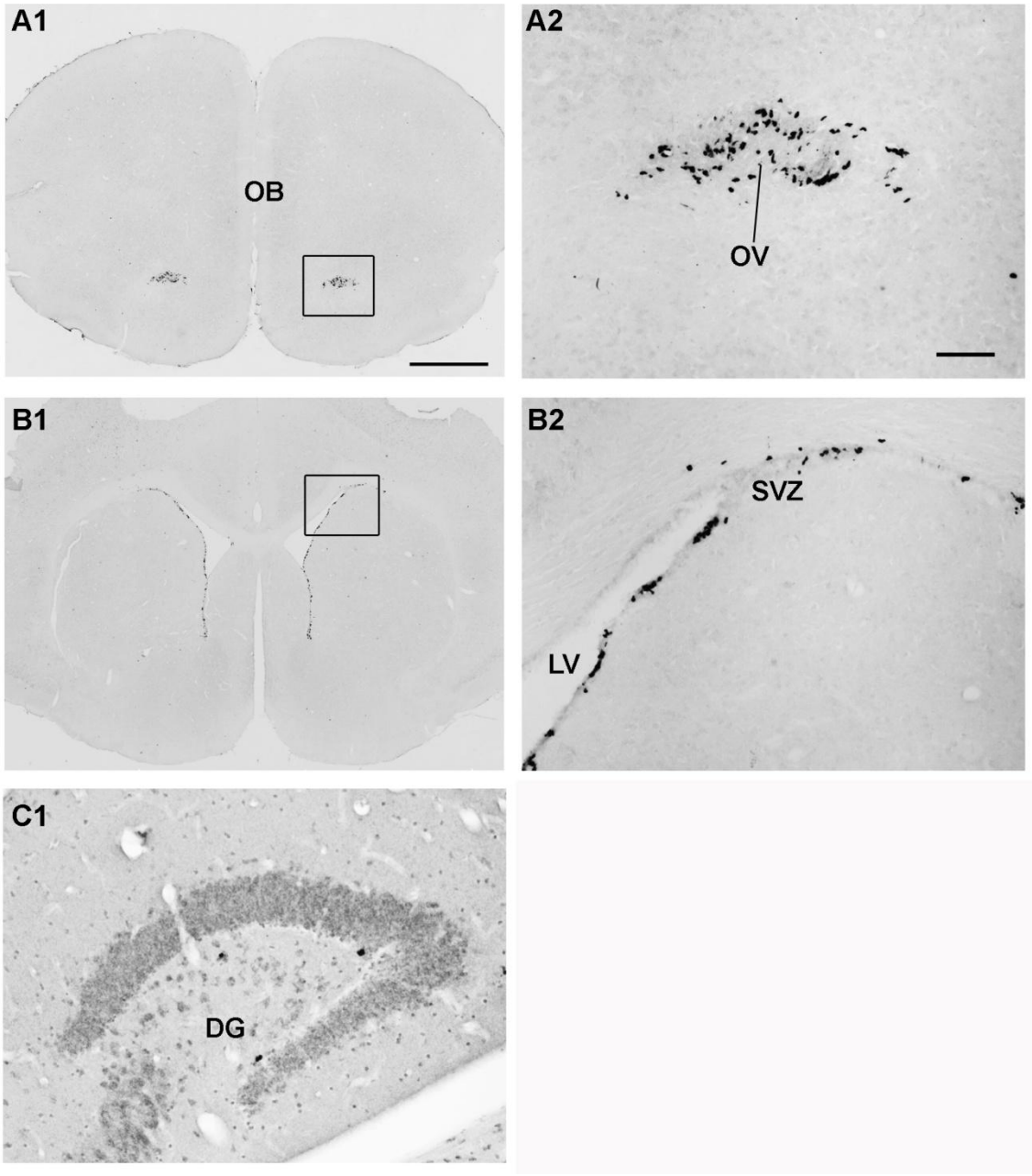


Plate 7.1: Brightfield photomicrographs of representative coronal sections showing BrdU-positive cell bodies in the brain of a female subordinate naked mole-rat. Areas enclosed in boxes (A1 and B1) are shown in higher magnification (A2 and B2). DG, dentate gyrus; LV, lateral ventricle; OB, olfactory bulb; OV, olfactory ventricle; SVZ, subventricular zone. Scale bar= 1mm for A1 and B1; 100µm for A2, B2 and C1.

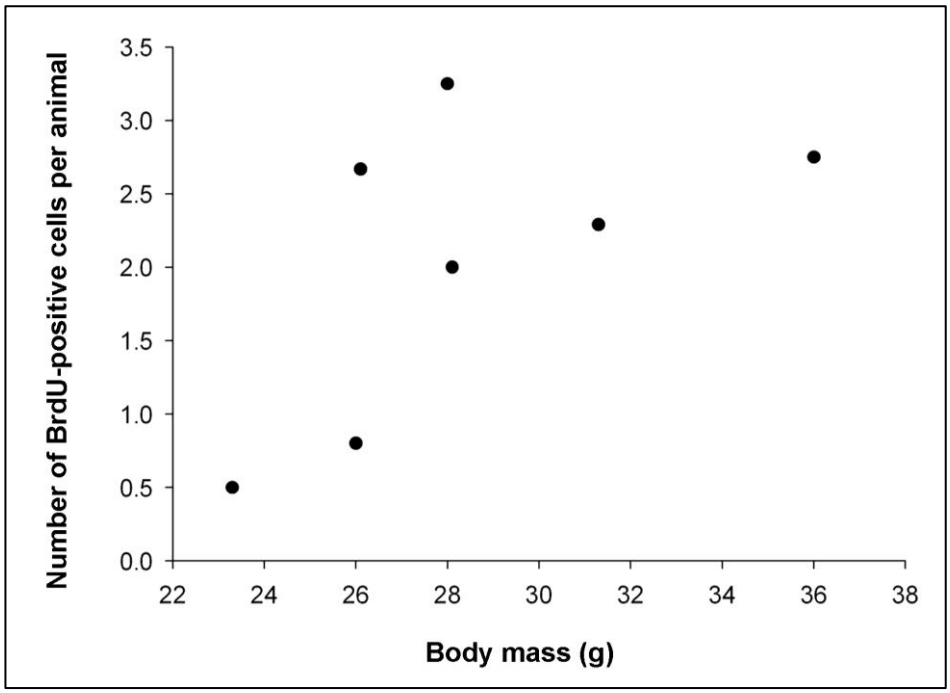


Figure 7.1: No correlation between body mass (g) and number of BrdU-positive cells

The distribution and quantification of DCX-immunoreactivity

Moderate numbers of DCX-positive cells were located around the OV of the OB (Plate 7.2A), SVZ (Plate 7.2B1-B2) and piriform cortex (PC) (Plate 7.2C1-C2). In the SVZ, DCX-positive cells were present tightly lining the ependymal layer of the SVZ (Plate 7.2B1-B2) and were hard to distinguish from glial cells. As a result, of DCX-positive cells were not counted in the SVZ. In the hippocampus, DCX-positive cells were located in the SGZ and the GCL of the DG. In the DG, the dendritic tree of DCX-positive cells extends through the GCL and into the ML (Plate 7.2D1-D2). There was a high degree of heterogeneity in the density of DCX-positive cells per mm between the four animal groups (Figure 7.2). But intriguingly, naked mole-rats had far greater density of DCX-positive cells than BrdU-ir cell bodies in the DG. For example, one female subordinate had a mean density of 83.8 DCX-positive cells whilst the highest number of BrdU-positive cells observed in the DG was only 5. There was no significant difference in the density of DCX-positive cells between the four animal groups, furthermore, there was no significant effect of sex or reproductive status; $F(3,17)= 0.912$, $p= 0.456$. Interestingly, there was a significant negative correlation between the mean density of DCX-positive cells per mm and body mass; $r= -0.467$, $p= 0.033$ (Figure 7.3). Thus, the heavier animals tend to have lower DCX densities and smaller animals tend to have higher DCX densities.

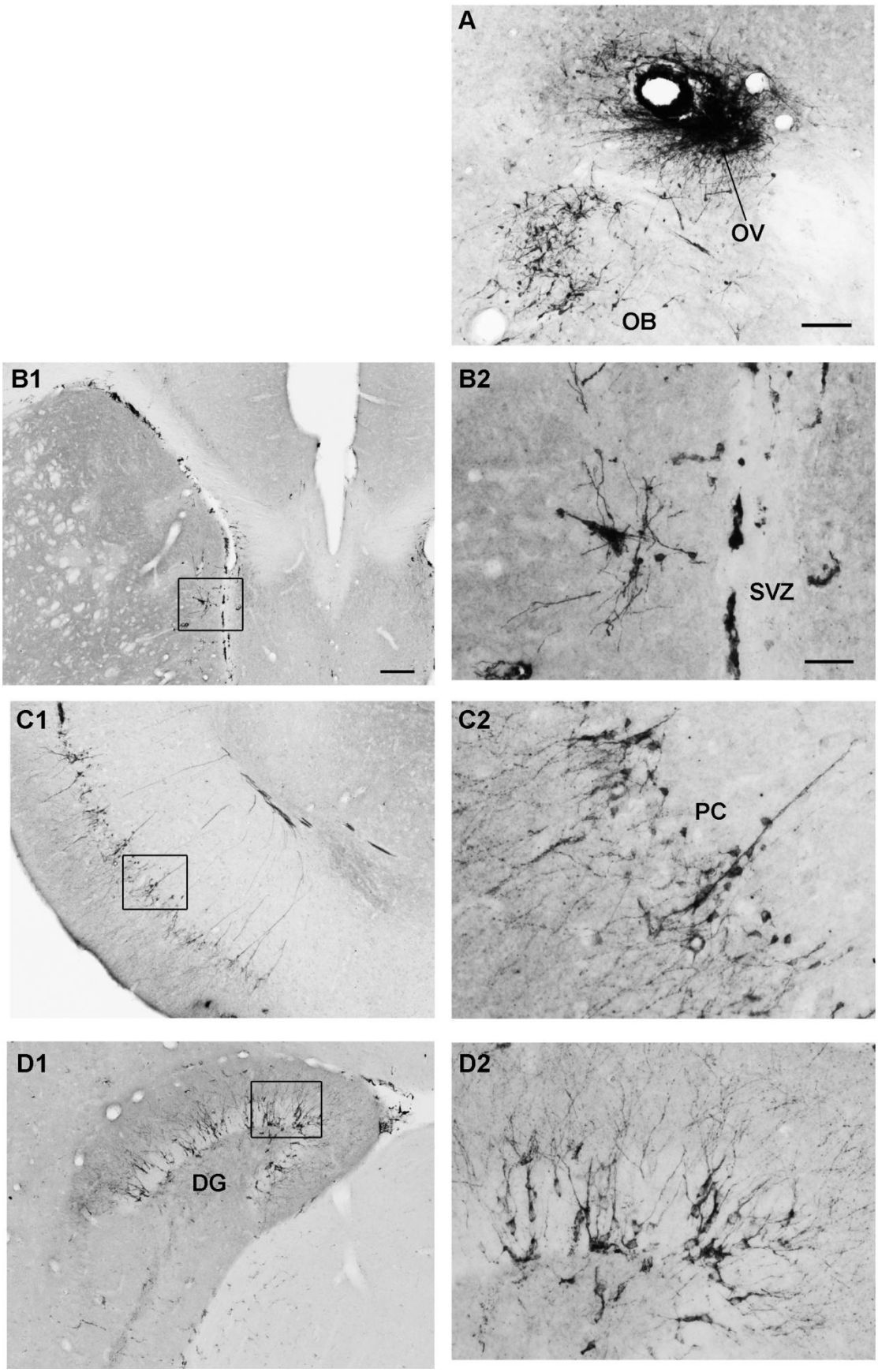


Plate 7.2: Brightfield photomicrographs of representative coronal sections showing DCX-positive cell bodies in the brain of a female subordinate naked mole-rat. Areas enclosed in boxes (B1, C1 and D1) are shown in higher magnification (B2, C2 and D2). DG, dentate gyrus;

OB, olfactory bulb; OV, olfactory ventricle; PC, piriform cortex; SVZ, subventricular zone. Scale bar= 100µm for A; 200µm for B1, C1 and D1; 50µm for B2, C2 and D2.

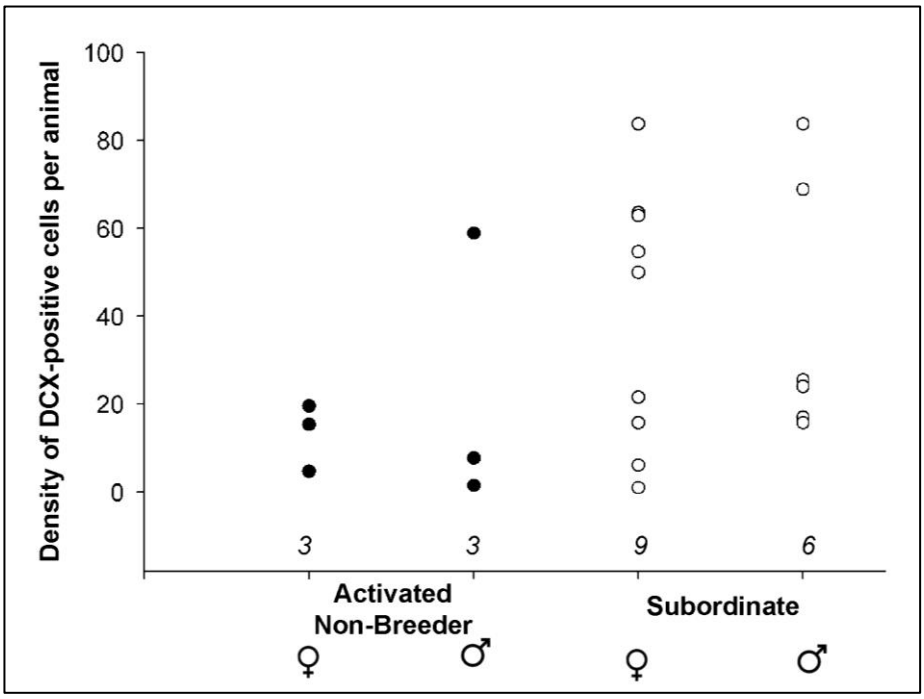


Figure 7.2: Density of DCX-positive cells per mm section of DG. Number of animals per group is noted at the bottom in italics. ♀ = female animal group; ♂ = male animal group. Number of animals per group is noted at the base in italics. Filled circles represent females; unfilled circles represent males.

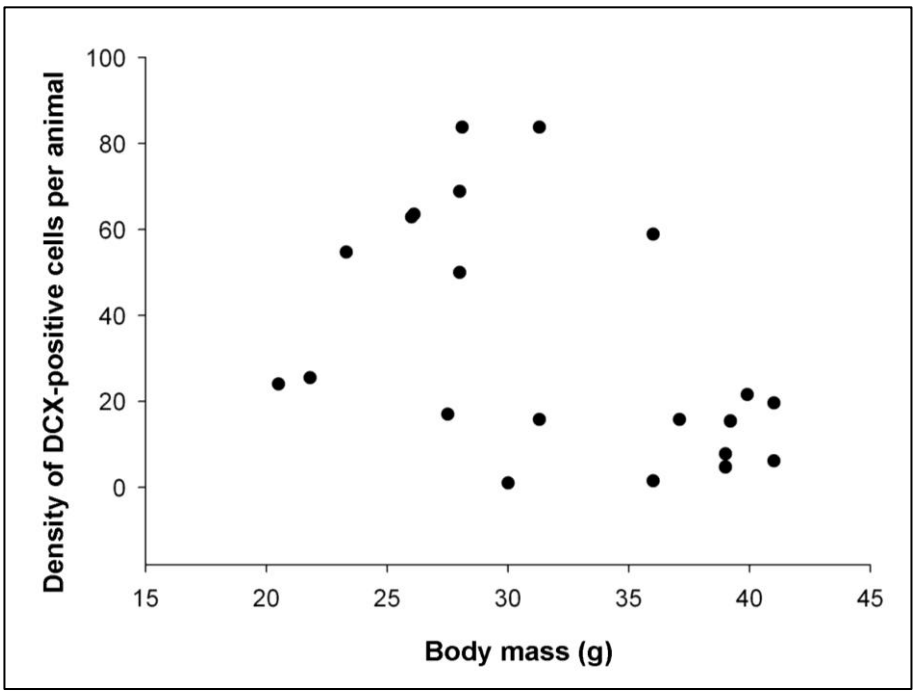


Figure 7.3: No correlation between body mass (g) and number of DCX-positive cells

DISCUSSION

BrdU is the most widespread marker of neurogenesis, and is the 'gold standard' by which all other markers in neurogenesis research are measured (Nowakowski *et al.*, 1989). However, by itself, BrdU does not give any indication of the phenotype of the marker cells. BrdU is a thymidine analog that incorporates itself into dividing cells during DNA synthesis where it will remain and be passed down to daughter cells following cell division. Thus, the successful marking of cells with BrdU first requires prior BrdU administration– either by injection or addition to drinking water (Wojtowicz and Kee, 2006). Unfortunately, the drawbacks of using BrdU include stressful injection procedure and uncertain penetration of the targeted cells with a uniform concentration of the compound (Kaplan, 1983; Gould and Gross, 2002). Therefore, endogenous markers, such as doublecortin (DCX), may be used instead to observe neurogenesis. DCX is a marker of developing, immature neurones. It is a protein required for normal neuronal migration in the developing cerebral cortex. It is microtubule-associated protein expressed in migrating neuroblasts, and is located in the periphery of the soma and its processes (Francis *et al.*, 1999; Gleeson *et al.*, 1999). Unlike BrdU, DCX labels the cytoplasm, not the nucleus of the cell (Brown *et al.*, 2003; Rao and Shetty, 2004). Both markers have different properties and have been found in both the adult RMS and the DG of the hippocampus of adult rodents

The results of this study indicates that naked mole-rats have a very low rate of adult hippocampal neurogenesis. The number of BrdU-positive cells in each DG section ranged from 0 to 5. The numbers of DCX-positive cells were higher, although still lower than most rodents (Amrein *et al.*, 2004). The first aim of this study was to determine the extent of adult hippocampal neurogenesis in naked mole-rats (fossorial animals living in burrows with complex spatial navigational requirements. A previous study has produced mixed results in assessing the neurogenic capacity of 'wild' animals, specifically, wood mice were found to have higher neurogenic activity whilst voles were found to have lower neurogenic activity than laboratory mice (Amrein *et al.*, 2004). In tests with laboratory animals in a complex environment (Kempermann and Gage, 1999), neurogenesis increases with novelty rather than with continued complexity. Thus, it seems likely that the high level of newly born neurones in wood mice is likely to be associated with the use of large, three-dimensional territories, which demands extensive and repetitive exploring by the wood mice.

Unfortunately, experimental rodent data about the functional relevance of hippocampal neurogenesis for learning and memory are often contradictory (Leuner *et al.*, 2006). Moreover, it is difficult to obtain a cause-effect relationship between neurogenesis and learning in a complex environment, other than just a correlation. A

possible shortcoming of many previous studies is that small laboratory test arenas might not be sufficient to trigger plasticity mechanisms that evolved for coping with natural large-scale orientation requirements typical for wild living animals. Although, in the wild, naked mole-rats live in long and complex burrow systems, in the laboratory, naked mole-rats are housed in much shorter and less complex burrow systems. Therefore, the naked mole-rats used in this study would not have had that much more spatial learning experiences than a typical laboratory rodent.

Whilst this simple laboratory environment may explain why the naked mole-rats in this study do not have high levels of neurogenesis, it does not explain the extremely low numbers of BrdU- and DCX-positive cells in the DG of naked mole-rats. In a previous study investigating hippocampal neurogenesis in different species of bats, bats were found to have absent or very low levels of hippocampal neurogenesis, despite the fact that bats had a similar high proliferation activity in the RMS to mice (Amrein *et al.*, 2007). This indicates that an absence of neurogenic capacity in the hippocampus of bats is not crucial for a variety cognitive functions, such as, spatial navigation and spatial reference memory in a complex environment. Thus, the low levels of neurogenesis found in species like bats and naked mole-rats indicates a lack of a simple correlation between environment and hippocampal neurogenic capacity.

Intriguingly, one crucial factor linking the low neurogenic capacity of naked mole-rats and bat (Amrein *et al.*, 2007) is longevity. Naked mole-rats and can live for up to 30 years, about 9 times longer than similar-sized mice (Buffenstein, 2008). They reach maturity in 6 months and only very old naked mole-rats (>28 years) show signs of age-associated pathologies (for example, muscle loss), but no signs of tumorigenesis (Edrey *et al.*, 2011). For at least 80% of their lives, naked mole-rats maintain normal reproductive and physiological functions with no obvious age-related increases in mortality rate. Their long lifespan is attributed to sustained good health and pronounced cancer resistance (Edrey *et al.*, 2011). Extended longevity is a common feature associated with group living, such as cave-roosting bats, mole-rats, and eusocial insects (honey bees, wasps, and ants), all these animals exhibit longer lifespans than expected on the basis of body mass. Humans also show missing or low ongoing proliferation in the hippocampus, though humans are not eusocial, there are certain parallels between long-lived eusocial species and human society: extended care of young, intergenerational transfer of information, and a division of labour, features that may enhance inclusive fitness by kinship and contribute to prolonged lifespan (Seress *et al.*, 2001; Boekhoorn *et al.*, 2006; Fahrner *et al.*, 2007). It would be interesting to analyse hippocampal neurogenesis in other species of mole-rats to determine whether low neurogenic capacity is shared amongst mole-rats.

An alternative explanation may be that sparse or missing hippocampal neurogenesis may be caused by down-regulation of mitogenic activity in the SGZ of the DG, especially since proliferation is regularly observed in the SVZ and olfactory bulb of bats and naked mole-rats. In this present study, the markers of neurogenesis (BrdU and DCX) were both used to detect the proliferation of new cells in the DG of naked mole-rats. However, neurogenesis is a process that consists of both cell proliferation (birth of new cells) and cell survival (new cells that survive to maturity). For future studies, it would to interesting to measure the rate the cell survival in naked mole-rats since another explanation for the low numbers of BrdU- and DCX-positive cells in naked mole-rats may be due to the long survival rates of new cells in the hippocampus. So, although naked mole-rats have low rates of hippocampal cellular proliferation (from the results of this present study), they can still have high hippocampal neurogenic capability if these new neurones have high survival rates.

Interestingly, there is a significant negative correlation between density of DCX-positive cells and body weight of the naked mole-rats. Within a naked mole-rat colony, a dominance hierarchy is formed whereby animals with a high body weight tend to have a higher dominance rank position (Clarke and Faulkes, 1997,1998). Dominance rank appears to be a good predictor of reproductive success since queens are the highest ranking females and are succeeded by the next higher ranking females in the colony (Clarke and Faulkes, 1997). The results of this chapter suggest that there is no significant effect of sex on the levels of hippocampal neurogenesis in naked mole-rats. This significant negative correlation between DCX-positive cells and body weight suggests that: heavier naked mole-rats will have a higher dominance rank and a lower density of DCX-positive cells, whilst smaller naked mole-rats will have a lower dominance rank and a higher density of DCX-positive cells. It is interesting that the smaller, more subordinate animals have a higher hippocampal neurogenic capacity than more dominant counterparts. Whether this difference is caused by the negative effects of dominance stress or physiological differences, the functional importance of this negative correlation remains to be determined.

Finally, the methodological considerations with the antibodies used in this study must be evaluated. In future investigations of neurogenesis in mole-rats, a multiple combination of neurogenesis markers should be used. Although BrdU is the most widely used neurogenesis marker, there are several major disadvantages to it. BrdU is not compatible with the investigation of wild animals under natural conditions, and the procedure of repeated injections of BrdU is stressful for the animals. Also there are uncertainties regarding the dose (Cameron and McKay, 2001; Gould and Gross, 2002), species-specific differences in the penetration of the blood-brain barrier (Kaplan, 1983), and the possibility of BrdU incorporation during cell repair (Gould and Gross, 2002).

These problems may have resulted in various contradictory findings in studies on environmental enrichment and hippocampal neurogenesis; some researchers found no effect of exposure to a learning task (van Praag *et al.*, 1999; Ambrogini *et al.*, 2000), whilst other researchers have found the complete opposite where new neurones born before a learning task survive and differentiate into new neurones (Gould *et al.*, 1999). It has been suggested that these differences may be due to the BrdU labelling method, whereby, no effect was seen in studies where tests were carried out during or after the BrdU labelling period, and an effect was seen when BrdU labelling was carried out before the tests. In a different study, the researchers only found an enhancement of hippocampal neurogenesis only when BrdU was administered 6 days prior to the beginning of spatial training (Epp *et al.*, 2007), indicating a critical period in the development of new neurones during which their survival may be altered by activation of the hippocampus. In this present chapter, the naked mole-rats were injected with BrdU only 24 hours before sacrifice due to logistical constrainsts, it is possible that we missed this critical period for peak BrdU labelling. Furthermore, there is evidence that the standard BrdU dosage (50mg/kg) is too low to label the majority of cell in S phase (Cameron and McKay, 2001). Thus, alterations in the BrdU methodology will be required for future studies on BrdU-immunoreactivity in naked mole-rats.

CHAPTER 9:

Conclusion

Mole-rats (of the Bathyergidae family) provide a unique taxonomic group for studying many aspects of neurobiology. In this family, ecological constraints have led to diverse social and reproductive strategies. Eusociality occurs in two species of Bathyergidae rodents; naked mole-rats, *Heterocephalus glaber*, and Damaraland mole-rats, *Fukomys damarensis*. The females of these two eusocial species are at the extreme end of the socially-induced infertility continuum whereby ovulation is physiologically blocked. This family provides the ideal model to glean insight into the neurobiological mechanisms of reproductive-suppression and eusocial behaviour.

This thesis focused on the neuroendocrine and neuroanatomical parameters of naked and Damaraland mole-rats to gain insights into the neurobiology of reproductive suppression, social behaviour and neurogenesis in eusocial animals.

Reproductive suppression

In 2003, the kisspeptin system was discovered and implicated in regulating reproduction (e.g. Seminara *et al.*, 2003), subsequent studies from numerous species have now provided a wealth of information supporting the model that hypothalamic kisspeptin directly activates the GnRH neurones via GPR54 to stimulate the HPG reproductive axis. So far, the study of kisspeptin as the key regulator of puberty has been focused on a limited number of relatively non-social laboratory animals, in which reproductive success is almost exclusively obtained through direct breeding. However, whilst kisspeptin appears to be crucial to maintaining fertility in many species, little is known about the 'rules' of HPG reproductive axis activation in animals with highly social reproductive strategies.

The reproductive phenotype of subordinate naked mole-rats resembles that of other hypogonadotrophic animals, such as subordinate marmosets, photoinhibited seasonally breeding animals and *Kiss1* or *Kiss1R* KO mice (Abbott *et al.*, 1988; d'Anglemont de Tassigny *et al.*, 2007; Revel *et al.*, 2007; d'Anglemont de Tassigny *et al.*, 2008). The hypothesis that hypothalamic kisspeptin contributes to the activation of the HPG axis when subordinate naked mole-rats are released from the presence of the queen was prompted by the evidence that it promotes the release of GnRH-1 required for onset and maintenance of reproductive functions in many other species (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Messager *et al.*, 2005; Navarro *et al.*, 2005). The distribution of kisspeptin-ir processes reported in chapter 3 is consistent with this peptide influencing GnRH-1 neurones at perikaryal and/or terminal levels. Nevertheless, the present findings show naked mole-rats to be different from previously studied species in various respects. Firstly, numerous kisspeptin-ir cell bodies are detected within each of the regional populations in all the groups. Secondly, the

kisspeptin system of naked mole-rats is independent from gonadal hormones and may be determined by their social status. This sexual monomorphism and independence from gonadal hormones lies in concert with the results from chapter 5 of this thesis and with previous findings on the naked mole-rats brain (Holmes *et al.*, 2007; Holmes *et al.*, 2008).

In the present study, the detection of numerous kisspeptin-ir cell bodies at all of the sites in all of the experimental groups was unexpected and raises questions about the state of activity and the functions of those neuronal populations. Thus, despite the presence of numerous kisspeptin-ir cell bodies in their RP3V and PVH, subordinates are unable to drive gonadotrophin release and activate their gonads. The greater number of kisspeptin-ir cell bodies in the RP3V and PVH of reproductively versus subordinate naked mole-rats may indicate that this population has crossed a critical threshold for HPG axis activation, indicating that reproductive control is at the level of responsiveness to kisspeptin release. Alternatively or additionally, there may be limiting factors such as the availability of kisspeptin receptors at specific sites. In this context, it should be noted that exogenous kisspeptin fails to release LH in photoinhibited female Siberian hamsters (Mason *et al.*, 2007).

It must be acknowledged that the immunohistochemical approach employed in chapter 3 may fail to detect signs of inhibited synthesis and/or release. Quantification of GnRH-1 mRNA expression will be needed to assess the dynamics of this system. Quantification of kisspeptin expression in this species by *in situ* hybridisation awaits development of an effective riboprobe; the probe that has been successfully applied to rat tissue (Kalamatianos *et al.*, 2008) does not function in naked mole-rats (Kalamatianos & Coen, unpublished findings). Similarly, it has not been possible to locate immunoreactivity for ERα in naked mole-rats using currently available antibodies (Kalló and Coen, unpublished findings). Nevertheless, the recent sequencing of the naked mole-rat genome (Kim *et al.*, 2011; Yu *et al.*, 2011) provides new resources. They will extend our ability to study this extraordinary species, most members of which stay reproductively suppressed throughout their long life, while remaining susceptible to activation by a simple intervention. Also, it would be useful to compare the expression of the kisspeptin receptor in GnRH-1 neurones of subordinate and reproductively-activated naked mole-rats to determine whether reproductive control is at the level of responsiveness to kisspeptin release.

In this thesis, RFamide-ir cell bodies are found in the MPOA of Damaraland mole-rats− a novel location in rodents. Also, breeding Damaraland mole-rats have a significantly higher number of MPOA RFamide-ir cell bodies than subordinates, with subordinates containing virtually RFamide-ir cells in the MPOA. There is no effect of gonadectomy or sex differences on the number of RFamide-ir cells. More substantially,

this present study has illuminated some technical problems regarding immunohistochemistry. In the past, there has been a lot of controversy regarding the cross-reactivity of kisspeptin antibodies with other RFamide peptides. Although a new kisspeptin antibody (from A. Caraty) has been created and tested rigorously for cross-reactivity, the lack of attenuation of staining in the MPOA after pre-adsorption suggests that there may be species-specific problems with this antibody. In the future, sequencing the Damaraland mole-rat kisspeptin gene will help us gain further insights into any species differences in the kisspeptin-10 amino acid sequence. This will enable us to establish *Kiss1* expression by *in situ* hybridisation for *Kiss1* mRNA and these expression studies will help us determine whether there is any overlap with the RFamide-immunoreactivity in Damaraland mole-rats. Nevertheless, the absence of these RFamide-ir cells in the MPOA of subordinate Damaralands mole-rats remains intriguing.

Extending our understanding of kisspeptin signalling in the brains of social species may clarify the diverse mechanisms by which kisspeptin, GnRH-1, gonadal hormones, other neuropeptides and social factors, act independently or collaboratively to mould the brain and behaviour of animals undergoing puberty. It would also be interesting to study the kisspeptin system of other cooperatively breeding animals, such as, marmoset monkeys, to determine whether kisspeptin system of other reproductively suppressed animals are also upregulated during the transition from subordinate to reproductive status. Extending our understanding of the neuroendocrinology of eusocial species may clarify how the HPG axis of 'non-traditional' species is regulated by social and hormonal factors.

Social behaviour

In this thesis I report a unique distribution of OT and AVP, and their receptor systems in the brains of eusocial Damaraland mole-rats. This study, along with previous studies on other mole-rat and vole species will contribute to our long-term aim of elucidating the neurobiology and molecular/biological regulation of OT and AVP systems and their evolution across the spectrum of eusocial, social and solitary mole-rat species. The shared presence of OTR binding in the NAcc of eusocial mole-rats and monogamous voles suggests that the NAcc OT system may confer sociality without necessarily conferring monogamy. Moreover, the presence of the NAcc OT system in both the eusocial naked mole-rats and Damaraland mole-rats may indicate the possibility of convergent evolution. During the evolution of Damaraland mole-rats from the common ancestor, NAcc OT system has evolved, then been lost through a line of solitary species, then re-evolved in the descendent Damaraland mole-rats (refer to figure 1.1 for the evolutionary tree of sociality in the Bathyergidae family). In the future, it would

be interesting to compare another animal family with diverse variations in social structures for other examples of convergent evolution. Since social species are dispersed across the mammalian phylogeny, indicating that sociality is likely to have evolved multiple times in mammals. Comparing the genetic basis of sociality in distant mammalian taxa provides an opportunity to examine whether evolution of complex social behaviour occurs through conserved mechanisms.

For the V1aR system, V1aR is not observed in the VP of in either eusocial mole-rat but is observed in the solitary Cape mole-rat. This indicates that the V1aR binding in the VP probably does not confer sociality. Rather, the loss of the V1aR VP system indicates that during the secondary evolution of eusocial Damaraland mole-rats, this receptor system has lost its ancestral function. Instead, social behaviour in Damaraland mole-rats may be conferred by other V1aR binding regions in the brain. In this thesis, I also report two novel locations for V1aR binding, the AVPV and NAcc. Given the evidence that eusociality has evolved twice in the family Bathyergidae, the presence of both OTR and V1aR binding in the NAcc may relate to parallel emergence of V1a and OTR and their respective ligands in the NAcc. The novel location of V1aR binding in the AVPV suggests that this V1aR system has independently evolved 14–17 million years ago as these animals diverged from the common ancestor and radiated north into Central Africa.

Neurogenesis

New neurones are continuously being generated in the hippocampal DG of adult animals. In this thesis, I present a new to study unique aspects of hippocampal neurogenesis: naked mole-rats. Eusocial naked mole-rats live in long and complex burrow systems, and spend most of their time foraging for underground tubers which are spread diffusely− a habitat where spatial learning and memory is an important skill for the survival. I report that naked mole-rats have a remarkably low rate of adult hippocampal neurogenesis in comparison to most rodents studied so far. However, previous studies on bats and humans have also discovered a remarkably low rate of neurogenesis in these animals. The lack of hippocampal neurogenic capacity in monkeys, humans and naked mole-rats suggests that hippocampal cellular proliferation is not crucial for a variety of cognitive functions, like spatial reference memory in a complex environment. Instead I hypothesise that longevity and group living in monkeys, humans and naked mole-rats may lead to low levels of hippocampal neurogenesis. Although the levels of hippocampal neurogenesis has not been studied in many long-lived and group-living species, this study indicates that the fundamental correlation between neurogenesis and cognitive function must be re-examined.

CHAPTER 10:

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